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A PHYSIOLOGICAL STUDY OF CRACKING IN STAYMAN WINESAP APPLES[1]

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INTRODUCTION

Cracking of fleshy fruits while still attached to the parent plant occurs in many cultivated species. In sweet cherry, apple, plum, peach, grape, strawberry, and some citrus fruits this kind of injury is sometimes so extensive as to result in great economic loss to growers. Rapid deterioration usually follows the exposure of the ruptured tissues to the air, and the injured fruit becomes worthless of or inferior grade. Losses of this nature amounting to as much as 75 percent have been observed by the writer in commercial orchards of sweet cherries, apples, and plums. Many fruits, on the other hand—such as sour cherry and some varieties of apple—seldom exhibit this phenomenon. In many fruits there are wide varietal differences in the extent to which cracking occurs under apparently similar environmental conditions.

The present paper deals primarily with cracking of the skin and underlying tissue in the fruit of the Stayman Winesap apple. Most of the observations and experiments were made in 1932–33 at the West Virginia University Experiment Farm at Kearneysville, and in 1933–34 at the Laboratory of Plant Physiology of the Johns Hopkins University.

In this study cracking has been observed extensively in only two varieties of apples, the Stayman Winesap and the York Imperial. In the Stayman Winesap cracking occurs largely on the cheeks of the fruit in the form of irregular breaks in the skin and underlying flesh (fig. 1). Individual cracks vary from almost invisible short slits to cracks several millimeters deep that extend in an approximately horizontal plane entirely around the fruit. There is no marked predominance of cracks in any particular plane in relation to the long axis of the fruit. Late in the growing season cracks originating near the fruit stem and extending outward in nearly straight meridional lines towards the cheeks are commonly observed. Cracks around the calyx basin are rare. In the York Imperial, cracks originating

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[2] For many helpful suggestions relating to these experiments, especially with reference to evaporativity as a climatic feature and to water relations in general, and for much help in interpreting the results and presenting them in this form, the writer wishes to express his appreciation to Prof. Burton E. Livingston, director of the Laboratory of Plant Physiology of the Johns Hopkins University. Helpful suggestions were received also from Dr. H. E. Knowlton, head of the Horticulture Department of West Virginia University; and acknowledgment is due Dr. A. B. Groves, of the Winchester Research Laboratory, Winchester, Va., for taking the photographs used in figure 1.

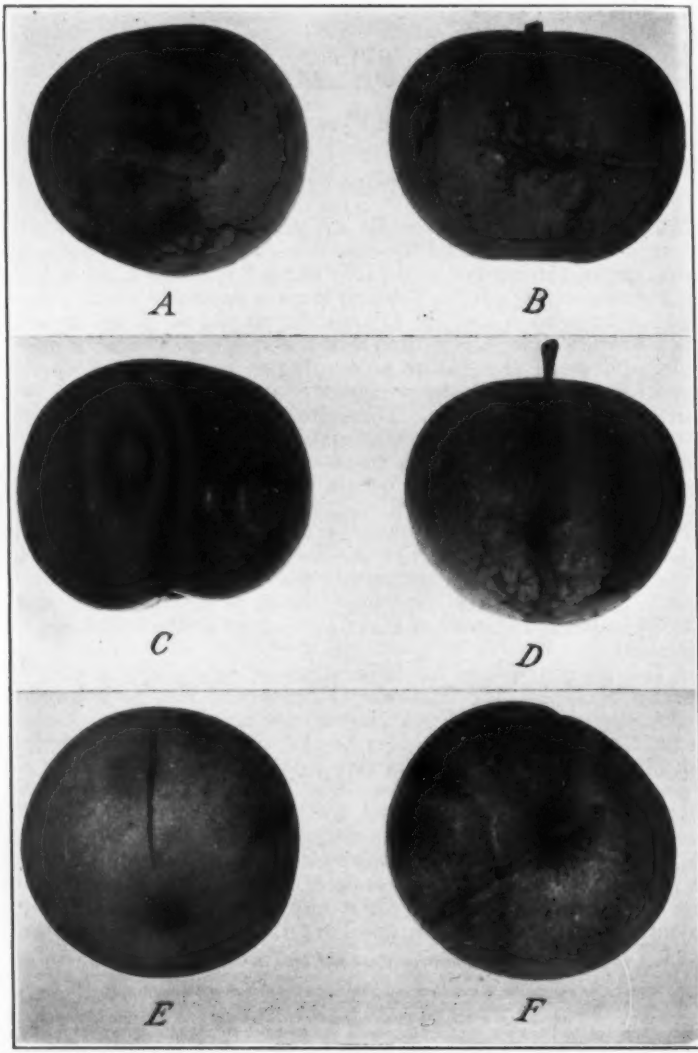

FIGURE 1.—Characteristic cracks in Stayman Winesap apples: *A*, Healed crack; *B*, *D*, and *E*, cracks originating in regions of russet; *C*, cracks originating in regions deformed by apple scab lesions; *F*, cracks originating in or near the stem depression.

at the calyx and extending like meridians toward the cheeks have been predominant in several hundred cracked specimens examined. Cracks in the stem cavity are also common in this variety, but on the cheeks of the fruit they are of much less frequent occurrence in the York Imperial than in the Stayman Winesap. In many specimens of Stayman Winesap, and in a few specimens of York Imperial, cracks that originated early in the season, when the apple was not nearly full grown, usually healed by suberization.

REVIEW OF LITERATURE

It has commonly been stated or implied that cracking of fruits is caused by a great and sudden increase in the water content of the soil, and that maintenance of a nearly constant soil-moisture content about the tree roots throughout the season of fruit growth should prevent cracking or greatly limit its severity. Discussing water relations of deciduous fruits in general, Chandler (*8, p. 165*)[3] states that "Certain injuries, such as cracking of the fruit, may result from a heavy irrigation late in its development, if growth has been checked by lack of water earlier."

Gardner, Bradford, and Hooker (*12, p. 83*) say that splitting is "most likely to occur shortly before maturity when rains follow a period of drought during which the fruit has been checked in its growth * * *. Heavy, late irrigation following a long dry season has the same effect." These writers suggest that a previous retardation of growth may render the fruit skin less able to expand rapidly in response to increased pressure within, thus predisposing the fruit to cracking when the growth rate is suddenly accelerated.

According to Heald (*17, pp. 101–101*):

> The rupturing of nearly mature, soft-skinned fruits, such as cherries, plums or tomatoes, when a rain follows a rather prolonged dry period is a fairly common phenomenon. These troubles have been proved to result from high sap pressure due to excessive water supply.

It is not clear whether the term "sap pressure" as used here refers to the vacuolar contents of the living cells in the region of the crack or to the contents of the tracheae which supply water to these cells; the causes of excessive pressure in the two cases might be very different.

Coit (*9*) discusses in some detail the development of splits in citrus fruits, with special reference to the navel orange. He says that these ruptures occur most frequently in the navel but are also found on the sides of the fruit, where they are associated with terratalogical cavities or "seams" in the skin. Regarding the causes of splitting in oranges, Coit states (*9, p. 328*):

> The most common theory in regard to the cause of splits is that an irregular water supply causing wide variations in the moisture content of the soil, produces a greater fluctuation in the growth of the interior than in the skin of the orange. Such a theory is quite reasonable, but such a cause should be regarded as contributory only, inasmuch as only a part of the fruit on any given tree will split.

Fawcett and Lee (*11*) point out that splits in citrus fruits are commonly associated with diseased tissues, such as lesions due to *Alternaria citri* or those accompanying the disease exanthema. In that disease the region of the oil vesicles in the rind is impregnated with gummy substances of the nature of pentosans, which, as these writers

[3] Reference is made by number (italic) to Literature Cited, p. 220.

suggest, may absorb water excessively when the water supply is plentiful and so cause rupture through abnormal swelling.

Boussingault in 1873 (*2*) demonstrated the ability of fruits of cherry, plum, pear, grape, and other horticultural forms to absorb water through the skin when submerged for a long period. Hartman and Bullis (*16*) reported that cracking of sweet cherries occurred in the Willamette Valley, Oreg., as a result of excessive water absorption by the fruit, either directly through the skin in wet weather or by way of the root system and vessels.

In extensive experiments with different periods and amounts of irrigation, Verner and Blodgett (*29*) were unable to observe any relationship between soil moisture and cracking in the three varieties of sweet cherries with which they experimented, although they tested extremes of soil moisture far beyond those usually found in irrigated orchards. Sawada (*21*), who experimented in Japan on the splitting of sweet cherries, likewise concluded that extremes of soil moisture played no direct part in producing the injury. He flooded the soil about the root system of a potted tree bearing mature fruit, but this drastic treatment failed to produce cracking.

Sawada was able to prevent cracking of sweet cherries on the tree by enclosing fruits in paraffined paper. By excluding rain by means of waterproof tarpaulins, Verner and Blodgett (*29*) succeeded in preventing the cracking of ripening fruit on large branches of sweet cherry trees in rainy periods, when severe cracking occurred on the exposed parts of the same trees. They concluded that cracking in this fruit was due mainly to direct absorption of rain water through the fruit skin. When fruits were held for a time under water, high rates of water absorption and pronounced cracking were both found to be concomitant with high concentrations of solutes in the expressed fruit juice, this concentration being regarded as a rough measure of the osmotic value of the cell sap.

Howard [4] states that he has observed splitting of sweet cherries in orchards at Mountain View, Calif., near San Francisco Bay, in periods of heavy fog without rain and without change in soil-moisture content through irrigation, and he suggests that this phenomenon may have been due to a sudden increase in the rate of water supply to the fruits, which might result from a decrease in the rate of water loss from the leaves. He says that root pressure might, under such circumstances, promote cracking by causing water to be forced into the fruit tissues from the vessels. Rixford (*20*) states that figs have been observed to split under conditions of high atmospheric humidity without rain or irrigation.

In the varieties of apples known as Cox Orange and Dunn, Campbell (*7*) and Goodwin (*13*) describe a russeting and cracking that led to great losses in the Nelson district of New Zealand. The latter writer, who describes the skin breaks of these apples as occurring only in conjunction with russeting or with lesions produced by the conothecium disease, considers the disorder as dependent upon general debility of the tree.

Sorauer (*23*) discusses the rupture of fleshy plant parts in general, considering the causal relations to be much alike in the many different forms of this disorder that he mentions, including fruit cracking in

[4] HOWARD, W. L. Correspondence. 1932.

cherry, plum, and grape, bursting of carrots and beets, and splitting of stems in kohlrabi, rape, bean, and potato. He says (*23, p. 322*): "All these phenomena have one characteristic in common—that they are initiated only when, after a considerable period of normal development, or still more after a previous dry period, an unusual supply of water is given suddenly."

According to Graebner (*14*), periods of drought result in the development of strengthening tissues, which usually appear first in the xylem and phloem. Mechanically strengthened cells have, as a rule, lost their ability to divide and most of their capacity to enlarge. If the water supply is greatly increased after a dry period, in the course of which the growth ability of some cells has been lost or has been greatly reduced, then the meristematic groups quickly resume growth, but corresponding growth cannot occur in the strengthened cells. Resultant differences in growth rates between contiguous mechanical and meristematic tissues may thus lead to excessive tensions, and rending of the mechanical tissue may ensue.

In a recent study by Frazier [5] it was found that tomatoes cracked most severely after heavy irrigation at the end of a prolonged dry period. Cracking was less severe in plots with frequent irrigation, which prevented excessive drying of the soil, and it was least severe in plots where the soil-moisture content remained low throughout the growing season. Shaded fruits cracked much less than those exposed to the sun.

Tracy (*25*) states that cracking of tomatoes may be prevented by enclosing the fruits when half grown in paper bags; but he points out that this treatment slightly impairs the flavor of the ripe fruit.

METHODS OF EXPERIMENTATION

The conditions that were studied in relation to cracking of apples may be divided into (1) external and (2) internal. The external conditions included those of weather (air temperature, air humidity, precipitation, evaporativity) and those of the soil, especially soil-moisture content. The internal conditions included rate of fruit enlargement, abnormalities of the peripheral region of the fruit, and freezing-point depression of the cortical tissue.

WEATHER DATA

Continuous records of temperature and relative humidity of the air were obtained throughout the growing season by means of a hygrothermograph installed in a standard United States Weather Bureau shelter located about 400 feet from the experimental block of trees. Temperatures and percentages of relative humidity, read directly from the automatic records at 2-hour intervals, were tabulated and employed as basic data. Temperature readings were used without modification, but corresponding readings of relative humidity and temperature were combined to give values of the vapor-pressure deficit.

Direct indices of the evaporating power of the air and of total sunshine intensity were obtained by means of Livingston porous porcelain atmometers (*18, 19*), both white and black spheres being used. The black sphere was of the new type, made of black porous porcelain.

[5] FRAZIER, W. A. A STUDY OF SOME FACTORS ASSOCIATED WITH THE OCCURRENCE OF CRACKS IN THE TOMATO FRUIT, 1933. (Ph. D. diss. Univ. Md.)

For 5 weeks in 1933, when the apples were thought most likely to crack, the black sphere was mounted on one end of an upright U tube of small bore (4 mm), the other end of which was joined to a vertical glass cylinder 3 cm in diameter and 24 cm high, open at the top, which served as a reservoir for supplying the atmometer with distilled water. Within this cylinder was a light, cylindrical glass float filled with air and sealed. To this float was attached a small vertical wire bearing a pen so adjusted that, as the float gradually descended in response to withdrawal of water from the reservoir, a continuous record was traced on a paper sheet borne by an upright, clock-driven drum. The slope of the tracing was thus a measure of the rate at which water evaporated from the standard black sphere.

Depth of rainfall, measured by a standard United States Weather Bureau rain gage, was recorded at intervals not exceeding 24 hours.

PROTECTION OF FRUIT FROM WETTING BY RAIN

Because water absorption through the fruit skin in periods when the latter is covered with a film of liquid water—as during and following times of precipitation—might conceivably be among the conditions that lead to cracking, some apples were covered to protect them from wetting by rain. This was accomplished by spraying with mineral oil emulsions or fish-oil soap, by enclosing apples in paper or cellophane bags, and by covering large branches of apples with waterproof tarpaulins in times of rain.

SOIL TREATMENT AND MEASUREMENT OF SOIL MOISTURE

The soil in the experimental block of trees is a clay loam of good fertility belonging to the Hagerstown series (*5*). Throughout the periods of observation on cracking there was always a moderately good stand of volunteer growth of weeds and grasses, which was undisturbed at these times.

In a region like West Virginia, which is subject to frequent and sometimes heavy summer rains, the experimental establishment of very marked differences in soil-moisture conditions in the field is very difficult, but an attempt was made to establish and maintain such soil-moisture differences among the experimental trees as were feasible. Throughout the periods when the likelihood of cracking was greatest, these differences were about as large as are ever found in orchards of this region. Special treatments with regard to soil moisture were as follows: (1) In 1933 the root system of one tree was deprived of the benefits of rains by diverting rain water from the root zone by means of waterproof tarpaulins supported on a wooden framework so arranged as to cover the soil area within and slightly beyond the spread of the branches, the intercepted water being discharged outside of that area. A marginal trench about 12 inches back from the outer edge of the tarpaulin covering served to prevent water seepage into the covered area. This trench was about 30 inches deep, extending at least 12 inches below the lowest roots intercepted, which were all cut. On October 6, when the fruit was nearly mature and when the moisture content of the upper 12 inches of soil under the tarpaulins had been reduced nearly to the wilting point, a flood irrigation equivalent to 4 inches of rainfall was given. (2) Early in the summer of 1933 several trees were mulched with

straw, the mulched area being somewhat broader than the spread of the branches. The covering, which was 4 to 6 inches deep after settling, was calculated to greatly retard water loss.

Soil samples were taken in 1933 at intervals of from 2 to 4 days between August 12 and October 7, embracing a period well in advance of, and including, that in which the most severe cracking is usually observed. At each time of sampling a cylinder of soil was taken, by means of a California soil tube (*27*), from the upper 6 inches and from the second 6 inches of soil. Samples were secured from three or more locations among the experimental trees, and the moisture percentages, calculated on the dry-weight basis, were averaged to give a single reading, which is taken to represent the soil of the experimental block as a whole.

FREEZING-POINT DETERMINATIONS

Freezing-point depressions of samples of apple tissue were ascertained from time to time by means of a Beckmann thermometer, a special tissue-cup method described by Verner (*28*) being used.

FRUITS UNDER WATER

Direct absorption of water through the fruit skin was studied for detached apples by sealing each stem and calyx opening with paraffin, weighing the fruits thus sealed, and then reweighing them after they had been kept under tap water for a time. Branches with fruits still attached were bent down and held with the fruit under water in 50-gallon barrels. By means of a steel tape graduated in millimeters the largest horizontal circumference of each apple was measured before and after this water treatment, and these measurements were compared with corresponding measurements of untreated fruits on adjacent branches.

RESULTS AND DISCUSSION

GENERAL OBSERVATIONS

Variability in Extent of Cracking

Field observations on apples have revealed marked differences in the incidence of cracking in the same orchard in different seasons, in different orchards in the same season, and in the same season among individual trees of the same orchard and branches of the same tree. For York Imperial and Stayman Winesap the extent of cracking in different orchards varied in 1932 from less than 5 to over 60 percent. At the experimental orchard near Kearneysville the percentage of fruit that cracked per Stayman Winesap tree varied from 33 to 65, the extremes appearing in adjacent trees. Two different branches of the same Stayman Winesap tree showed, respectively, 31 and 70 percent of cracking. In several cases one fruit of a spur was badly cracked while a second fruit of the same spur, with almost the same exposure and with the same outward appearance, was unaffected. Thus, while weather conditions exert a major influence in the cracking of apples, it is apparent that physiological conditions within the tree or fruit, not directly related to current weather features, are also influential.

Nature and Rate of Crack Formation

In periods when weather conditions were such as to warrant the prediction that cracking would soon occur, many fruits were examined with a hand lens at frequent intervals, and it was thus possible to detect incipient cracks when they were much less than a millimeter long and to observe the regions in which they occurred and the manner in which they enlarged and multiplied. It was evident that cracks generally appeared first in restricted areas, especially in areas of russeting, sunburn, or high skin coloration. It appeared as though the peripheral tissues were exceptionally weak in such regions or the tissue strains were more pronounced there than elsewhere. Localization was usually very definite, and it was often possible to detect a dozen or more minute breaks within an area of a square inch. Enlargement of cracks sometimes continued for several days, especially if evaporation rates were low.

Many incipient cracks apparently originated at hypertrophied lenticels of the cracking area (fig. 2). If the cracks remained small until after the weather became drier, cork was formed in the minute fissures and a russeted area might thus result. The russeting here considered may therefore have resulted, at least in part, from lenticel hypertrophy, incipient cracking, and subsequent cork formation.

Several writers have concluded that lenticel hypertrophy may be caused or promoted by greatly retarded transpiration from the plant, accompanied by a plentiful water supply to the regions of hypertrophy. Thus the formation of the corky or mealy excrescences that mark lenticel hypertrophy on young stems and branches of such trees as cherry and elder are attributed by Sorauer (*23*) to temporary retardation of foliar transpiration in periods of high atmospheric humidity. Schilberszky (*22*) concluded that hypertrophy of lenticels in apple fruits is related to an excessive water supply in the soil. Devaux (*10*) considered lenticel formation to be intimately connected with excessive moisture supply in underlying tissues, and Swingle (*24*) found that lenticel hypertrophy in stem cuttings of willow was directly correlated with their water supply under conditions of negligible evaporation. The proliferation that constitutes lenticel hypertrophy may decrease the extensibility of the neighboring peripheral cell layers and lower their mechanical resistance to being torn apart; and if that be true lenticels might be expected to mark the weakest points, at which rupture should begin whenever peripheral tissue strains become sufficiently excessive.

As to the formation of large cracks, numerous observations showed that it usually required at least several hours for these to develop. Thus, in one instance, the development of a crack 11 mm in length and about 1 mm in maximal depth required 3 hours, counting from its first clearly visible appearance as a mere slit about a millimeter long. Some cracks developed more rapidly; one attained a length of about 60 mm in 6 hours after its inception. A representative sample of the larger cracks showed successive lengths of 11, 18, 22, and 43 mm at 48-hour intervals.

RELATION OF NATURAL ENVIRONMENTAL CONDITIONS TO CRACKING

Explanation of Graphs

Data on natural fluctuations in soil-moisture content, rainfall, atmometric evaporation, and air humidity in relation to cracking of apples on the experimental trees in 1933 are summarized in figure 3.

FIGURE 2.—Hypertrophied lenticels, cracks, and russet development in a Stayman Winesap apple. × 3.

The smallest subdivision on the abscissa represents a 2-hour interval. The 5-week period considered (Sept. 3 to Oct. 8) covers the latter part of the period of fruit enlargement. The ordinate scale represents the index values of the respective environmental features.

The two upper graphs present the general march of soil-moisture percentage on the dry-weight basis. In the arbitrary scale of ordinates at the left, unity represents a moisture percentage of 4, which is the scale factor for these two soil-moisture graphs.

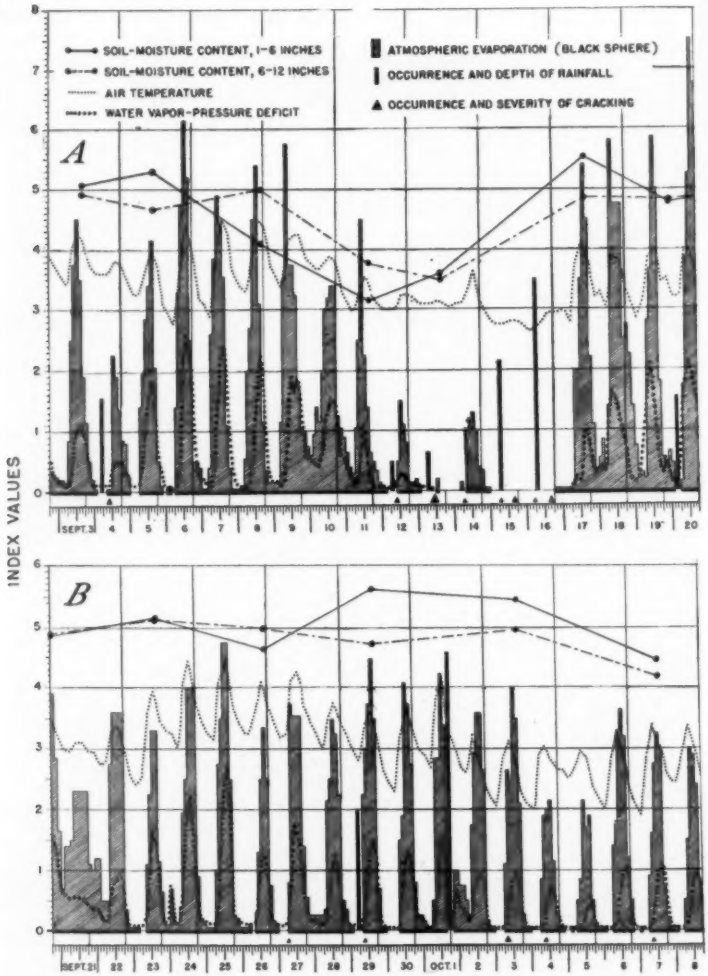
SOIL-MOISTURE CONTENT, 1-6 INCHES
SOIL-MOISTURE CONTENT, 6-12 INCHES
AIR TEMPERATURE
WATER VAPOR-PRESSURE DEFICIT
ATMOSPHERIC EVAPORATION (BLACK SPHERE)
OCCURRENCE AND DEPTH OF RAINFALL
OCCURRENCE AND SEVERITY OF CRACKING
A
B
INDEX VALUES
SEPT. 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
SEPT. 21 22 23 24 25 26 27 28 29 30 OCT. 1 2 3 4 5 6 7 8

FIGURE 3.—Relation of cracking in Stayman Winesap apples to fluctuations in environmental conditions Sept. 3 to Oct. 8, 1933. See text for complete explanation.

The march of air temperature is shown by the narrow dotted line, which is derived from the automatic tracing of the thermograph. The scale factor for air temperature is 20; that is, unity on the general ordinate scale at the left represents 20° F.

The wider dotted line shows the march of the water-saturation deficit of the air. This deficit is taken to represent the current evaporating power of the air with the wind and radiation factors removed. For this feature unity on the ordinate scale corresponds to a water-vapor pressure deficit of 8 mm of a barometric mercury column.

The occurrence of measurable rainfall is indicated by vertical black bars. The height of any bar shows the total depth of rain accumulated in the 24-hour period ending at 6 p. m. on the day referred to. Duration of rainfall was not recorded; therefore only a single bar is shown for each day with measurable precipitation, and all these bars have the same width, which is arbitrary. The scale factor for rainfall is 0.2 inch or approximately 0.5 cm.

Fluctuations in the rate of evaporation from the standardized black porous-porcelain atmometer sphere, exposed in the open, are shown by the hachured stepgraph of figure 3. This graph is plotted for every 2-hour period, as though it consisted of vertical bars, each 2 hours in width. The height of each bar or step represents the total accumulated water loss for its period. The scale factor for evaporation is 2 ml per 2-hour period; that is, unity on the general ordinate scale corresponds to water loss from the instrument at a mean rate of 1 ml per hour for the corresponding 2-hour period. It is at once obvious that the intensity of this feature fluctuated greatly and that there were occasional periods without measurable evaporation. For such periods this graph naturally coincides with the base line. The horizontal extent of each hachured block has been indicated by broadening the base line, and intervals without measurable water loss from the atmometer are clearly shown by the absence of the broad base line. Periods without evaporation, or periods of very slow evaporation, are of special interest, as will later appear.

Observations on cracking of the fruit were made at intervals of 2 or 3 hours in the daytime, but no observations of this sort were made at night. For each observation cracking was recorded only for the number of fruits that had been noted as uncracked at the last preceding observation. Consequently the total number of observed fruits used as a basis for these percentages became smaller as the season advanced. Naturally, the most susceptible fruits were the first to crack when external conditions favored this injury, and later observations on occurrence and degree of cracking were confined to fruits that had not already been recorded as cracked. In the present connection all cracks were considered as equal, and no attention is here given to additional cracking of fruit previously recorded as showing cracks.

Because of these considerations the percentage values derived from the observational counts should be adequately weighted or re-evaluated before being employed quantitatively in this part of the study, but no satisfactory way of so modifying them has been found. Therefore the cracking record presented in figure 3 is in very general terms as (1) slight, (2) moderate, and (3) severe; representing, respectively, about the following values: (1) from 1 to 5 percent, (2) from 6 to 15 percent, and (3) more than 15 percent. These three relative degrees of cracking are indicated, respectively, by small, medium-sized, and large black triangles placed just beneath the base line; the abscissal position of each triangle shows when the corresponding outbreak of cracking was observed.

Relation of Air Temperature and Soil Moisture to Cracking

There appears to be no correlation at all between air-temperature fluctuation and the occurrence of cracking, so far as may be judged by a study of the graphs of figure 3.

The fluctuations of soil-moisture content shown in figure 3 exhibit no relation to cracking. These fluctuations were relatively slight, and it is safe to suppose that the soil-moisture content of the first 6-inch and the second 6-inch depth was, in the 5-week period considered, never low enough to approach closely the value of the wilting coefficient of this soil. The wilting coefficient of the soil of the experiment orchard at Kearneysville may be estimated as about 10 percent on the gravimetric basis and according to Veihmeyer's (*26*) interpretation of this apparently critical value. The depth to which the present records refer (0 to 12 inches) is estimated to have included the region occupied by more than three-fifths of the roots of these trees.

Veihmeyer has shown (*26*) that the rate of water utilization by prune and peach trees with which he experimented in southern California was independent of the amount of moisture present in the soil as long as this amount was above the wilting coefficient. He found that the rate at which water was removed from the soil by a tree appeared to be determined primarily by the evaporating power of the air and the magnitude of the total leaf surface of the tree. If this is generally true of fruit trees it is hardly to be expected that changes in soil moisture within the limits usually met with in nonirrigated orchards—that is, above the wilting point and below field saturation—should affect changes in the water balance of the tree sufficient to produce cracking.

Relation of Rainfall to Cracking

Since soil-moisture content shows no apparent relation to cracking, wetting of the soil by rain is not to be regarded as among the influences that promoted this injury. If the occurrence of rain really promoted cracking in this 5-week period its influence must have been exerted in some other manner than through an increase in the supply of water to the tree roots. During rainy periods the foliage and the apples themselves were, of course, more or less completely covered with water, and it is conceivable that direct absorption of water through leaf epidermis and fruit skin may have been directly or indirectly influential in promoting the tissue strains that lead to cracking.

An indirect way in which the occurrence of a rainy period following a drier one might promote cracking is through sudden lowering of the transpiration rate, for transpiration must be practically prevented as long as the transpiring surfaces are covered with water. But in periods of little or no evaporation, transpiration must be very slow whether or not rain occurs.

However these interdependent variables, rain and evaporativity, may operate in general, a study of the graphs in figure 3 makes it clear that, although the outbreaks of cracking usually were preceded immediately by rain, this was not always so. Thus in several instances (on Sept. 27 and Oct. 3, 4, and 7) cracking was observed when there had been no recorded rain for from 36 hours to several days, and in other instances (Sept. 20 and Oct. 1) cracking failed to occur in spite of heavy rains.

Relation of Atmometric Evaporation to Cracking

Figure 3 shows, for the 5-week period represented, a very clear and consistent relation between occurrence of cracking and occurrence of very low rates of evaporation from the black atmometer sphere. Cracking was never observed except after a period of slow evaporation, and nearly every period of 6 hours or more without recorded evaporation was accompanied or immediately followed by cracking. On October 7 cracking followed two 4-hour periods without evaporation, the two periods being separated by a 2-hour interval with very low rate. On the other days when cracking occurred it was preceded in every instance by at least 6 hours without measurable water loss from the atmometer. As has been mentioned, although prolonged periods without atmometric evaporation were, in a number of instances, concomitant with periods of rainfall, there were also periods of cracking that obviously were not related at all to the occurrence of rain. Examination of figure 3 shows that these periods of cracking without antecedent rain (Sept. 27 and Oct. 3, 4, and 7) in every case were immediately preceded by from 6 to 8 hours of little or no measurable atmometric evaporation. Conversely, as has just been pointed out, on October 1 no cracking was observed during or following a rain of 0.92 inch, when the evaporation rate was zero for only 4 hours, then rose abruptly; nor was there any cracking following the lighter rain of September 20, when, also, the period of slow evaporation was short. The nonoccurrence of cracking in the prolonged periods of low evaporation on September 5 and September 30 may, perhaps, be accounted for partially or wholly by the fact that in each case there had been considerable cracking on the preceding day; presumably the apples currently most susceptible to this injury had already cracked.

Relation of Water Vapor-Pressure Deficit of the Air to Cracking

As would be expected, the graphs of vapor-pressure deficit have almost exactly the same form as the corresponding ones of atmometric evaporation. The period from September 13 to 16 (fig. 3) includes two intervals for which this deficit has zero value and both of these are without measurable atmometric evaporation. Also, both show records of severe cracking. The remaining intervals without measurable evaporation from the black sphere, as these are represented in figure 3, show very low deficit values of 1 mm or less. All the outbreaks of cracking recorded in this figure were preceded by 4 or more hours with vapor-pressure deficit below 1.6 mm.

A fairly close relationship was found to exist between relative humidity, or relative-humidity deficit, and cracking. The most extensive cracking in any 24-hour period in 1933 took place on August 24 in a 10-hour period when relative humidity was recorded as 100 percent. This period followed a rainfall of 4.25 inches. In 1932 approximately 40 percent of the crop of Stayman Winesap apples in the experimental orchard were damaged by cracking in a 38-hour period on October 17 and 18, when the recorded relative humidity at no time was below 99 percent. The total rainfall for these 2 days was 2.07 inches, but the soil had a high water content before the rain began. A heavier rain on October 5 and 6, totaling 2.85 inches

but accompanied by humidity for the most part well below 90 percent, caused no cracking. Similar instances of apparent association of cracking with prolonged high air humidity, either with or without rainfall, were observed many times in 1932 and 1933 and in several varieties of apples, including Stayman Winesap, York Imperial, Northern Spy, Wealthy, and Yellow Transparent. At no time during these 2 years was cracking observed except during periods of 8 hours or more when relative humidity was above 90 percent.

ORCHARD EXPERIMENTS WITH MODIFICATION OF NATURAL ENVIRONMENTAL CONDITIONS

Modification of Soil-Moisture Content

In an attempt to obtain somewhat greater extremes in soil-moisture content than ordinarily occur in the region in which these experiments were conducted, the soil over the root system of one tree was protected from rain for several weeks by means of waterproof tarpaulins, as has been noted. This shelter was erected on August 31, 1933, when the average soil-moisture percentage for the upper 12 inches in the experimental block was 18.1. It was removed on October 6, when the 12-inch index for the sheltered soil had decreased to 12.4 percent and that for the rest of the block was 17.4 percent. Immediately after the removal of the tarpaulins the soil area that had been covered was given a flood irrigation equivalent to 4 inches of rainfall. About 10 hours later the 0- to 12-inch soil-moisture content for this area was found to have increased to 20 percent. This sudden increase in soil moisture failed to produce any cracking. Similar irrigation of one tree in 1932, without previous use of tarpaulins but following prolonged dry weather, had also failed to produce cracking. A heavy straw mulch had no apparent influence on cracking, although such a soil cover retards water loss from the soil while it permits free penetration of rain water.

Between August 31 and October 6, 1933, there was cracking of the fruit on the tree with the tarpaulin-sheltered root system when cracking was common among other trees of the experimental block. Since the soil about the roots of this tree was protected from being wetted by rain throughout that period, it is clear that wetting of the soil was not required to produce cracking. Whether or not a sudden and sufficiently pronounced rise in soil-moisture content following a period of very dry soil may lead to cracking of apples under certain conditions is, of course, still an open question; but the present study furnishes no clear evidence that wetting of the soil was causally related to the cracking here recorded. Under the natural and the artificially modified soil conditions of this study, it appears that very slow atmospheric evaporation was probably the immediate external cause of cracking.

Fruits Protected from Wetting by Rain

With the possibility in mind that direct water absorption through the apple skin in a period of rain may sometimes be a contributing cause of cracking, the following experiment was carried out. A large

branch, bearing about 50 apples, was covered with a waterproof tarpaulin when rain occurred, the cover being removed when rain had ceased and the rest of the foliage was no longer wet. In a period of no atmometric evaporation, which was accompanied by rain, three of these protected apples cracked in spite of the fact that their skins were superficially dry. Although the number of apples involved in this test was not sufficient to furnish general evidence as to whether the prevention of wetting reduced the percentage of cracking, the result clearly supports the evidence already discussed that wetting of the fruit surface by rain is not, at least in general, necessary to cause cracking. The presence of rain water on the fruit may, however, favor cracking when other influences also are favorable to it.

Attached Branch Under Water

On September 30, 1932, a large Stayman Winesap branch bearing fruit without any cracks was bent down into a barrel of water so that all the apples and most of the leaves and small branches were submerged. This experiment continued for 4 days, and in that time severe cracking occurred. In the 4-day interval there were no periods of very low evaporation rates and no change of soil-moisture content other than the usual slow decrease due to ordinary evaporation and root absorption. At the same time no cracking of normally exposed fruits was observed, either on the same tree or on others. Measurements of maximal horizontal circumferences of the submerged apples, and of those on an adjacent untreated branch of the same tree, showed that in the 4-day period the mean rate of enlargement of the submerged fruits was much greater than that of the exposed ones. Details of this experiment are presented in table 1.

The fruits of both groups were originally of about the same size. The maximal enlargement percentage for submerged fruits (2.52) is almost double that for exposed fruits (1.34). The average enlargement percentages are 1.47 (submerged fruits) and 0.93 (exposed fruits), the former being about 1.58 times the latter; that is, the apples on the branch under water increased their maximal horizontal circumferences, on an average, about 58 percent more rapidly than did the apples on the exposed branch.

Twenty of the thirty-four submerged fruits cracked in the 4-day interval considered, but there was no cracking among the exposed fruits. The submerged fruits may be classified in three groups, according to their rates of enlargement, nos. 1 to 11 being considered as enlarging slowly, nos. 12 to 22 as enlarging at an intermediate rate, and nos. 23 to 34 as enlarging rapidly. The average length of cracks in the slowly enlarging group was 0.75 cm (2 out of 11 apples cracked), in the median group 6.0 cm (7 out of 11 apples cracked), and in the rapidly enlarging group 7.6 cm (11 out of 12 apples cracked).

TABLE 1.—*Enlargement rates of Stayman Winesap apples still attached to tree, some on branch submerged in water and others normally exposed on adjacent branch of same tree, together with records of cracking for submerged fruits, September 30 to October 4, 1932*

[No cracking of exposed fruits occurred. Data are arranged in ascending order of enlargement rates in each group]

Apple no.	On submerged branch			On adjacent branch	
	Sept. 30, original circumference	Oct. 4		Sept. 30, original circumference	Oct. 4, increase in circumference
		Increase in circumference	Total length of all cracks		
	Centimeters	*Percent*	*Centimeters*	*Centimeters*	*Percent*
1	22.95	0.22	(1)	22.95	0.65
2	22.05	.45	(1)	22.70	.60
3	24.05	.63	(1)	22.15	.68
4	22.95	.65	1.1	21.30	.70
5	22.70	.66	(1)	21.55	.70
6	19.65	.76	7.1	19.65	.76
7	23.40	.85	(1)	24.30	.82
8	23.10	.86	(1)	24.15	.83
9	22.30	.90	(1)	23.60	.84
10	22.10	.90	(1)	23.40	.85
11	22.75	.90	(1)	22.20	.90
12	24.35	1.23	11.8	21.70	.92
13	19.55	1.28	(1)	21.12	.94
14	23.10	1.30	(1)	20.50	.97
15	23.20	1.30	1.1	20.00	1.00
16	23.00	1.30	1.4	24.65	1.02
17	21.65	1.39	(1)	24.05	1.04
18	22.50	1.51	5.7	23.90	1.04
19	22.50	1.51	17.6	23.20	1.08
20	21.80	1.60	(1)	23.00	1.09
21	20.70	1.69	14.8	22.75	1.10
22	23.50	1.70	14.5	22.60	1.10
23	21.50	1.86	(1)	22.85	1.10
24	24.05	1.87	1.5	22.55	1.11
25	21.10	1.90	6.9	21.35	1.17
26	23.20	1.94	12.0	21.20	1.18
27	21.90	2.05	10.0	23.70	1.26
28	20.55	2.20	9.9	23.70	1.26
29	24.50	2.26	3.2	23.35	1.29
30	22.10	2.26	9.6	23.05	1.30
31	21.90	2.28	6.3	22.90	1.31
32	21.65	2.31	5.8	22.30	1.34
33	21.50	2.33	4.9		
34	23.85	2.52	21.5		
Average	22.40	1.47	4.9	22.57	.93

[1] No cracking.

While the water treatment seems, in general, to have increased the incidence of cracking, especially when it also increased the rate of fruit enlargement, the severity of cracking was apparently influenced by conditions not taken into quantitative account, presumably by variable internal characteristics of the fruits at the time of the experiment. The occurrence of cracking seems to be dependent not so much upon the rapid enlargement of the fruit as a whole as upon extra rapid enlargement of a restricted region of the tissues. The following chance observation made on September 16, 1933, may bear on this suggestion. In an apple with more than a third of its volume involved in a soft rot extending from the surface to the core and so advanced that the diseased portion was reduced to a mass of soft pulp, severe cracking had taken place on the uninfected part. This cracking must have been entirely independent of enlargement of the fruit as a whole.

Leaf Injury and Defoliation

In a large block of Stayman Winesap trees near Shenandoah Junction, about 3 miles east of the Kearneysville orchard, which was used for most of this study, the crop of 1933 showed very little cracking (less than 0.5 percent), while 40 percent of the same year's crop at the Kearneysville orchard was cracked. The trees of these two orchards were of the same age (14 years), both orchards were on the same type of soil, and weather conditions must have been virtually the same for both. The two orchards were noticeably different in that the Kearneysville trees had been partially defoliated or their foliage had been somewhat injured by spray materials, whereas the foliage in the other orchard was unusually dense and healthy. More of the fruits at Kearneysville were partially russeted, probably due largely to spray injury, and more of them were sunburned or highly colored, due to greater exposure to sunlight. All of these conditions, i. e., russeting, sunburn, and high coloration appear to be associated with increased cracking. Similar relationships between foliage conditions and cracking were seen among individual trees and branches in the experimental block at Kearneysville, where the highest percentages of cracking were associated with the highest percentages of leaf injury or with the sparsest foliage.

Figure 4, *A* and *B*, shows the general appearance of densely foliaged branches with little cracking as compared with sparsely foliaged branches with severe cracking. Data of a more quantitative nature bearing on this relation between foliage density and severity of cracking were secured by comparing three representative branches of about the same size but showing different degrees of cracking with respect to the total fresh weight of the leaves. One branch (*A*, at Shenandoah Junction) showed no cracking and the other two (nos. 2–22 and 4–9, at Kearneysville) showed, respectively, slight and severe cracking. The apples on all three of these branches were virtually free of russet and other visible skin injuries. Table 2 presents these data.

Table 2.—*Comparative data on number and diameter of fruit and fresh weight of leaves as related to the occurrence of cracking in apples*

Branch no.	Apples on branch	Average diameter of apples	Total fresh weight of leaves	Degree of cracking
	Number	*Inches*	*Grams*	
A	103	2.75	1,710	None.
2–22	72	2.25	1,518	Slight.
4–9	76	2.75	828	Severe.

Attached Apples Enclosed in Brown-Paper Bags

In the autumn of 1932 about 200 Stayman Winesap apples on three experimental trees were bagged and these were observed from time to time, observations being made, at the same times, on a similar number of comparable fruits, previously tagged, on these same trees. Cracking of the unbagged fruits severe enough to furnish a useful measure of the effects of bagging did not occur until the bagged apples had been enclosed for 3 to 4 weeks—time enough for pronounced morphological and physiological alterations to take place. Therefore the bagged

FIGURE 4.—*A*, Part of a Stayman Winesap tree with dense foliage and little cracking, near Shenandoah Junction; *B*, part of a Stayman Winesap tree with sparse foliage and severe cracking, near Kearneysville, W. Va.

apples probably differed markedly from the controls long before the latter showed much cracking. When about 30 to 50 percent of the controls had cracked, only about 2 to 6 percent of the protected apples had cracked. This is shown by records taken on October 20, presented in table 3.

TABLE 3.—*Cracking of bagged and unbagged Stayman Winesap apples*

Tree no.	Bagged apples			Unbagged apples	
	Apples	Days bagged	Cracked	Apples	Cracked
	Number	*Number*	*Percent*	*Number*	*Percent*
3-12	110	30	6.4	90	54.4
4-11	50	27	6.0	50	30.0
4-12	50	20	2.0	50	30.0

By harvest time the enclosed apples had developed the solid yellow ground color characteristic of this variety at full maturity, but they were devoid of any trace of red overcolor. It is reasonable to suppose that protection for many weeks from the direct rays of the sun might so influence either the osmotic values or the structural characteristics of the apple as to make it less inclined to crack. The effects may, therefore, have been due to prolonged shading, just as dense foliage seems to have reduced the tendency toward cracking.

OIL-EMULSION SPRAYS

In 1932 extensive tests were conducted with different concentrations, and with different times of application, of fish-oil and mineral-oil sprays, on the supposition that the oily films left on the fruits might retard or prevent absorption of water through the skin and so reduce cracking. No effects upon cracking were observed, however. When sprayed and unsprayed apples were subsequently tested under water it was found that the sprayed fruits although distinctly oily to the touch, absorbed water about as rapidly as did the untreated ones. Apparently the oil sprays used had failed to reduce the permeability of the skin to water.

FRUITS REMOVED FROM THE TREE AND SUBMERGED IN WATER

The evidence thus far presented leads to the conclusion that wetting of the soil by rain or irrigation was not a considerable influence in promoting cracking in the experimental orchard. The other possible influence of rain (i. e., the maintenance for a time of a more or less complete liquid water layer over the fruit and foliar surfaces) is not shown by figure 3 to have been influential in producing cracking; but when an attached branch bearing fruit and leaves was kept under water for 4 days this treatment was very effective in producing cracking, especially in fruits in which the water treatment accelerated enlargement. The experiment with the submerged branch was supplemented by some additional experiments with apples removed from the tree and kept under water.

After the cut end of the stem and the calyx opening had been sealed with paraffin each apple was weighed and all were placed in water, where they were held for 2 days. They were finally wiped and reweighed, the weight gain of each was computed, and the degree of cracking shown by each was recorded as the total length of all its cracks. Data from this experiment with mature apples removed from the trees are shown in table 4.

TABLE 4.—*Weight increase and cracking in mature Stayman Winesap apples kept under water for 2 days, Oct. 24 to 26, 1931*

[Apples are arranged in the ascending order of their weight gains]

Apple no.	Original weight	Gain in weight	Total length of all cracks	Apple no.	Original weight	Gain in weight	Total length of all cracks
	Grams	*Percent*	*Centimeters*		*Grams*	*Percent*	*Centimeters*
1	207	0	(1)	11	255	2.35	3.75
2	222	.90	(1)	12	286	2.45	(1)
3	195	1.03	(1)	13	244	2.46	4.25
4	243	1.24	(1)	14	219	2.74	14.75
5	158	1.26	2.75	15	166	3.01	4.50
6	241	1.66	4.25	16	195	3.08	4.25
7	172	1.75	1.00	17	246	3.25	11.25
8	172	1.75	1.75	18	237	3.38	(1)
9	198	2.02	2.25				
10	220	2.27	1.25	Average	215	2.32	3.11

[1] No cracking.

These results are, in general, similar to those shown by the orchard test with growing apples still attached to the tree when the branch bearing them was kept under water for 4 days (table 1). The water treatment of detached apples resulted in much cracking, and most of the badly cracked fruit showed relatively large gains in weight.

As in the orchard experiment on cracking of attached apples under water, it is clear that this 2-day water treatment of mature, detached fruits did produce cracking, that it was more apt to do so when the rate of enlargement was rapid than when it was slow, and that severity of the cracking produced generally was greater for those fruits that enlarged more rapidly. Since the cut end of the stem and the opening of the calyx region were sealed, it is evident that weight gain must have been due to water absorption through the skin. This observation furnished additional evidence that such direct absorption is to be expected whenever the apple skin is kept externally wet for a sufficient time. The apples that failed to crack also absorbed water, and one of these (no. 18) showed the most rapid absorption of all.

EXPERIMENTS ON VASCULAR PRESSURE AND CONDUCTION

A few experiments were performed with reference to possible relations between the occurrence of cracking and conditions that might influence the supply of water and other substances to the fruit. These are briefly described below.

Vascular Sap Pressure in Attached Branches

Mercury manometers were connected to small glass cylinders filled with water and fastened over the cut surfaces where tip portions of attached branches had been removed. These manometers invariably

showed maintained pressure deficits in the attached branches. It was thus apparent that the hydrostatic pressure of the vascular sap generally was considerably less than the gas pressure of the surrounding air. This was true even when cracking of the fruits was in progress, but the pressure deficit was smaller in periods of low evaporation rates than at drier times. Degrees of suction amounting to from 143 mm of a mercury column to 400 mm were observed. The method used is of course inadequate to show maximal pressure deficits, and it seems safe to suppose that the deficits recorded were generally smaller than the actual current deficits in uninjured trees or even in the cut branches some distance back of the cut surfaces. We may, in any event, conclude that vascular hydrostatic pressure in a branch when apples were cracking generally was considerably less than the current barometer reading.

These manometer experiments indicate clearly that there was no general vascular pressure excess in these trees, which might, as some writers seem to think, act to force water into the fruits at times of cracking. There was no evidence of positive excess pressure derived from the roots and transmitted throughout the tree, such as seems to have been postulated whenever the term "root pressure" has been employed in the elaboration of hypotheses concerning cracking of fruits and other phenomena connected with plant-water relations.

Excessive Water Pressure Artificially Produced in Branches

Some unsuccessful laboratory attempts were made to cause apples to crack by forcing water under excessive pressure into the cut basal ends of branches bearing fruit. The cut end of the branch, where the latter had been detached from the tree, was tightly sealed into the large opening of a tubulated filter flask containing water enough to cover the cut surface to a height of about 5 cm, and a Schrader valve, such as is used in automobile tires, was securely fastened to the tubulature by means of a bit of rubber tubing and a wire ligature. Then a hand-operated compression air pump was connected to the valve and air was pumped into the flask to increase the gas pressure in its upper portion and so force water into the branch. Pressures of from 5 to 10 pounds were employed. No cracking of the fruit resulted from this treatment, in which branch and fruit were exposed to the air of the laboratory. This experiment was not tried with branch and fruit surrounded by water-saturated air.

Within a few minutes after pressure had been applied to the system, water dripped rapidly from surfaces where ends of lateral twigs had been cut off, and more slowly from cut midribs of leaves; but no exudation appeared at surfaces where fruit stems had been cut. These tests lasted from 30 minutes to 3 hours. The results would be more significant if the artificially produced excessive vascular pressure had been applied for a longer time and if transpiration had been prevented, as by keeping leaf and fruit surfaces continuously wet.

Dye Solutions Absorbed Through Cut Ends of Branches Still Attached to Tree

Aqueous dye solutions were fed into the cut ends of small branches from 1 to 2 cm in diameter, and dye penetration into nearby fruits was observed. Both acid fuchsin and methyl blue were used with

equally satisfactory results. The solution entered under the influence of vascular-pressure deficit, no excess pressure being applied. The color contrast of either dye with the whitish flesh of the apple was quite striking, the dyed regions standing out clearly when the fruit was cut open. The dyes appeared in the regions of the vascular bundles and to a greater or lesser extent in contiguous meristematic tissues. Methyl blue was detected in very small veinlets close under the skin.

A striking feature of the penetration and accumulation of these dyes was their consistently greater concentration in regions subjacent to abnormal peripheral tissues, such as russeted areas, apple-scab lesions, and old cracks. Although sap movement through the vessels was probably somewhat more rapid and perhaps somewhat more extensive than was indicated by dye accumulation in the cell walls, yet there is no reason to suspect that the dye ever moved more rapidly than the sap that carried it, and the final presence of more dye in one region than in another surely means that sap had moved into the more intensely stained parts more rapidly than to other parts that showed less dye accumulation. It therefore appears that sap flow to the modified or injured peripheral regions just mentioned was much more rapid than to uninjured peripheral tissues, and this apparently indicates, in turn, that transpiration was more intense from the injured tissues than from other peripheral regions of the fruit. Baker (*1*) found that transpirational water loss through the corky tissue of russeted regions of apples was considerably greater than through normal epidermis and cuticle. Since the tissues just beneath the corky regions do not wither as long as the apples are on the tree, but remain turgid and healthy, this observation implies a more rapid movement of water into those regions.

From what has just been said it may be concluded that, except when transpiration is almost stopped, russeted, cracked, or otherwise locally injured peripheral tissues of the apple fruit transmit water from vessels to periphery more rapidly than do uninjured tissues, whose resistance to transpirational water loss is apparently greater; i. e., for the cells of the injured tissue both entrance of water and water loss generally are more rapid than for the cells of corresponding uninjured tissue. Many students have presented evidence in support of the general observation that larger bundles and more vessels develop in regions adjacent to rapidly transpiring surfaces than in other regions; in some unknown manner it appears that exceptionally rapid flow of sap through a developing bundle causes the formation of more vessels, or of more efficient ones, than would have been formed had the rate of sap flow been much slower. On the basis of this proposition it may be supposed that vascular resistance to sap movement is apt to be exceptionally low in those bundles that lead to the injured regions of the apple. If such is the case, vascular sap may be supposed to reach those regions with somewhat less hydrostatic pressure deficit than prevails in other regions, especially in times of very slow or negligible foliar transpiration, when vascular-pressure deficit must be exceptionally low throughout the whole tree. In periods of rapid transpiration the suction (or even liquid traction) arising therefrom should act to retard cell absorption everywhere. These considerations of logical possibilities may be assembled to furnish a hypothesis that

may explain, at least in part, why injured portions of apple periphery proved to be more susceptible to cracking than other portions, and why outbreaks of cracking and the occurrence of the most severe cracking were confined to periods when the general transpiration rate was unusually low.

INTERNAL CONDITIONS OF FRUIT IN RELATION TO CRACKING

General Relations Between External and Internal Influences

As has been pointed out, cracking of the fruit is of much more common occurrence in some apple varieties than in others. When cracking is prevalent in an orchard some trees usually are affected much more than others and some branches often are affected more than others on the same tree. Even on those fruiting branches exhibiting the most severe cracking when external conditions tend to promote this injury, some apples usually fail to crack. In the present study some apples were always found to be uninjured at harvest time, even on trees that exhibited high percentages of cracking.

It is therefore evident that cracking of individual fruits must be determined not alone by the occurrence of favorable weather conditions but also by some internal conditions effective within the fruit at times when the weather is favorable to cracking. The phenomenon of cracking is to be considered as the result of some combination or concatenation of circumstances, some of which may be traced almost directly to environmental fluctuations, while others appear to result from antecedent physiological processes of nutrition, growth, and development within the apple tree or within the fruit itself. In apples the formation of a crack shows that the fruit tissues involved, and perhaps other tissues also, were, when cracking occurred, unusually susceptible to the current cracking influence of the environmental complex, and it shows just as clearly that the conditions of the environment were such as to permit or facilitate crack formation in tissues exhibiting this particular kind and degree of susceptibility. Consequently the problem of the causation of cracking cannot be considered satisfactorily in terms of environmental conditions alone or in terms of internal conditions alone; both external and internal influences need to be taken into account.

The preceding sections of this paper deal mainly with cracking as it appeared to be related to environmental conditions, although attention is given to some internal conditions: e. g., vascular sap pressure and injured or modified regions of the fruit periphery. The present section deals with some observations on additional internal conditions: viz, abnormalities of peripheral tissues and the freezing-point depression of cortical tissues.

Abnormalities of Peripheral Tissues

As has been noted, cracking is less likely to occur on sound apples than on those with some abnormality of the peripheral tissues. Cracks are found much more often on portions of the fruit periphery that are marked by russeting, by scab lesions, or by sunburn than on other portions of the fruit. This point is illustrated by figure 1. Both in 1932 and 1933 the lesions that appeared earliest in any outbreak of cracking were predominantly on these modified parts of the

fruit surfaces. In 1933, 88 percent of the cracks formed on the fruits of one tree were directly associated with russeted skin, sunburn, or scab spots. The remaining 12 percent were in fruit surfaces that appeared otherwise to be perfectly healthy and unmodified. Cracks on otherwise sound fruits most often were on the cheek that was most exposed to sunlight. It should be emphasized, however, that cracking in apparently normal, uninjured peripheral regions has shown the same relationships to environmental fluctuations as was shown by cracking in the regions of modified periphery. Cracking in abnormal regions of the fruits as observed in these experiments is not to be regarded as a special form of the injury dependent on the abnormalities themselves, that is, the abnormalities merely render affected portions of fruits more susceptible to the injury than normal portions when environmental influences tend to promote cracking in both.

Freezing-Point Depression of Fruit Tissues

The observation that cracking was much more apt to occur on the most exposed side of an apple, especially when the skin and adjacent tissues on that side were modified by high coloration, sunburn, or russeting, leads to the suggestion that the turgor pressure in the tissues underlying the modified or injured portion of the periphery might be greater than that of corresponding tissues underlying unmodified peripheral regions of the same fruit. Brooks and Fisher (*3*) found that on very hot summer days the sunny side of an apple had a temperature from 10° to 16° F. higher than the shaded side. Juice expressed from the most exposed quarter of the fruit generally showed greater freezing-point depression than juice similarly expressed from the opposite quarter, and this difference was greater as the exposure of the first quarter was more pronounced. This was taken to indicate higher osmotic concentration in the cell sap of the tissues on the exposed side, which might be due, for example, to the higher temperature or to the more intense insolation on that side during periods of sunshine.

In a later paper (*4*) the same authors reported that exceptionally high osmotic values were indicated for tissue from the sunburned side, when one side was so injured; also that there seemed to be positive correlation between evaporation from a Livingston standardized white-sphere atmometer and the osmotic value of juice from apples in different parts of the tree, both being greatest in the position of greatest exposure to sunshine. A similar effect of seasonal conditions on the crop as a whole is suggested by Caldwell's (*6*) analyses of apple juice, which show, for many varieties, on unusually high sugar content in seasons with temperature and sunshine considerably above normal.

By means of the tissue-cup freezing-point method (*28*) many comparisons were made between sample cups from different regions of the same apple, especially between tissue samples from regions underlying unmodified skin areas and other tissue samples from regions underlying areas of high coloration, sunburn, or russeting. In some instances the more susceptible region of the apple tested had already cracked; that is, tissue underlying one or more newly developed cracks was compared, with reference to freezing-point depression, with tissue underlying an intact area of the same apple. After making the usual correction necessitated by supercooling, each freezing-point

depression was translated into terms of osmotic concentration in atmospheres by reference to the published table of Harris and Gortner (*15*). It should be emphasized that this index refers to the sample cup of tissue as a whole, not to its cell sap alone. It represents the capacity of the tested tissue to retain water against the influence of drying agencies, and it embraces not only the resilient pressure of stretched cell walls (which tends to squeeze water out of the cells), but also the osmotic value of the cell sap and of the liquid held in the walls themselves, as well as the imbibitional values of cell colloids and cell walls (which together act to retard water loss).

Results derived from 52 different apples of the Stayman Winesap variety, picked from eight different trees, are presented in table 5. Two values are shown for each apple, derived, respectively, from two tissue-cup samples taken from opposite sides of the fruit axis, with the longitudinal dimension of each cup parallel to the latter. The tissue of each cup was thus derived from a region opposite the middle portion of the apple core and about half-way between the core and the cheek periphery on that side. One of each pair of cup samples was taken from beneath a cheek area that showed modification of some sort, while the other sample was taken from beneath unmodified cheek periphery. Five kinds of visible modification or injury of peripheral tissue were recognized: (1) highly colored skin, uncracked; (2) sunburned skin, uncracked; (3) russeted periphery, recently cracked; (4) russeted periphery, uncracked; and (5) recently cracked periphery otherwise apparently unmodified.

It is seen at once that the tissue samples derived from beneath modified or cracked areas of the fruit periphery showed significantly higher osmotic values, in almost every test, than were shown by the samples derived from beneath unmodified areas. Since highly colored, sunburned, or russeted areas of the fruit surface have been found to be exceptionally prone to crack, this remarkable osmotic relation lends definite support to the supposition that cracking may result from excessive enlargement or excessive tissue pressure in localized subperipheral regions of the fruit cortex; for, other conditions being the same, regions of exceptionally high osmotic value should absorb water, and therefore enlarge, more rapidly than neighboring regions of lower osmotic value. The data of table 5 therefore indicate a reason why cracking is most likely to occur over regions of the cortex that are superficially marked by high coloration, sunburn, or russeting. The averages for the several sections of table 5 lead to the same conclusions as are reached from more detailed study. For every group of comparisons the average for the unmodified is smaller than for the modified areas.

TABLE 5.—*Osmotic values (in atmospheres) of tissue samples from opposite sides of the same apple (Stayman Winesap) computed from freezing-point depressions obtained in 1933 by means of the tissue-cup method (28)*

Tissue from beneath skin of high coloration, uncracked			Tissue from beneath sunburned periphery, uncracked			Tissue from beneath russeted periphery, recently cracked			Tissue from beneath russeted periphery, uncracked			Tissue from beneath recently cracked but otherwise unmodified periphery		
Date	Tissue beneath modified periphery	Tissue beneath apparently normal skin	Date	Tissue beneath modified periphery	Tissue beneath apparently normal skin	Date	Tissue beneath modified periphery	Tissue beneath apparently normal skin	Date	Tissue beneath modified periphery	Tissue beneath apparently normal skin	Date	Tissue beneath modified periphery	Tissue beneath apparently normal skin
Sept. 22	14.20	13.36	Oct. 13	26.55	18.40	Sept. 30	26.67	17.80	Sept. 22	19.48	13.84	Oct. 7	16.96	15.52
	22.60	13.96		23.44	17.92	Oct. 10	22.12	17.20		13.72	12.76		18.64	17.08
Sept. 29	18.52	20.80	Oct. 15	24.75	19.48	Oct. 13	17.32	16.60		13.00	11.32	Oct. 10	20.68	17.68
Sept. 30	18.40	17.32		23.68	18.76		18.76	16.36	Sept. 25	24.52	16.48		18.04	19.12
	25.95	16.24		25.59	17.80	Average	21.22	16.99		21.28	19.00	Oct. 11	18.04	17.50
	21.52	17.20		23.56	17.44				Sept. 30	22.48	17.68		17.44	17.80
Oct. 16	19.00	16.00	Average	24.59	18.30					18.76	18.76	Oct. 13	19.24	18.16
	19.72	16.72							Oct. 4	19.72	17.08		18.88	16.24
	19.12	15.76							Oct. 5	21.88	15.76		18.88	15.40
	19.12	16.00								18.40	16.36	Average	18.53	17.17
	20.08	17.56							Oct. 11	19.84	19.24			
Oct. 24	19.60	17.32								19.84	19.24			
	19.12	16.84								19.36	17.68			
	18.28	17.92							Oct. 15	22.12	20.32			
	20.20	19.24							Oct. 24	20.08	17.32			
Oct. 25	20.08	17.72							Average	19.63	16.86			
	23.32	20.44												
	22.25	22.84												
Average	20.06	17.51												

Pronounced localization of high tissue pressure is clearly indicated, and it seems likely that differences within the same apple might be found to be even greater than those shown in table 5 if it were possible to employ smaller tissue samples in freezing-point determinations of this kind. An attempt was made to chart the pressure variations somewhat more in detail through the use of 5 tissue cups from each apple instead of 2, the positions for sampling being symmetrically arranged around the fruit axis. The results of these more elaborate tests were in excellent agreement with those set forth in table 5. A representative set of data from five samples is shown in figure 5. The small circles represent the positions of the tissue cups studied, and the numerical values within these circles are osmotic values like those given in table 5. Apparently there was a tissue-pressure gradient in the subperipheral cortex of the fruit, between a high pressure underneath the more exposed peripheral area and a much lower pressure in the corresponding region on the opposite side of the fruit axis.

When the several local osmotic pressures found by employing 4 or 5 tissue samples from the same apple were averaged, to give an approximation of the mean-tissue pressure of the whole fruit, it was found that this mean value showed no relation to cracking. Some apples with low means were badly cracked while some with much higher means showed no cracking. It is therefore indicated that the internal conditions that favor or hinder cracking are not to be studied by means of freezing-point values derived from whole apples or from pulp or juice obtained by grinding or pressing the entire fruit.

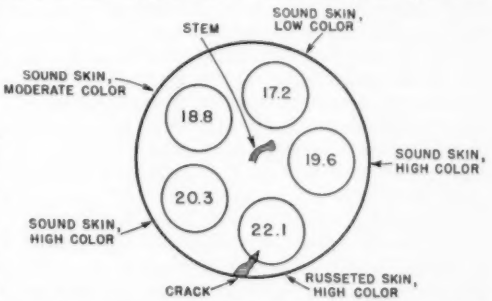

FIGURE 5.—Graphic representation of osmotic values, in atmospheres, of tissue samples from different portions of one Stayman Winesap apple.

There was apparently no relation between a tendency to crack and the absolute tissue pressure of the subperipheral cortex; cracking might be associated with a local tissue pressure of only 15 atmospheres in one apple, while a local pressure of 20 atmospheres or more might be found in another apple that was still sound. It seems that cracking must be related, in general, not to the local subperipheral pressure itself but to the magnitude and distribution of the pressure gradient just mentioned. In short, one of the main internal conditions leading to crack formation appears to be the occurrence of an excessive tissue pressure in a restricted area of the fruit cortex, accompanied by much lower pressures in neighboring regions.

SUMMARY AND CONCLUSIONS

This paper deals with cracking of the peripheral tissues of Stayman Winesap apples, which is considered in relation to current environmental and internal conditions. The discussions and views presented

are based on (1) observational studies carried out on cracking under natural conditions in an experimental orchard at Kearneysville, W. Va., (2) orchard experiments in which some environmental conditions were artificially modified, and (3) special laboratory studies.

Sudden, great increase in soil-moisture content has been suggested by some writers as a probable major factor contributing to cracking of fruits in general, but the observations and experiments recorded in the present paper fail to furnish any evidence in support of this hypothesis. Pronounced fluctuations in soil-moisture content, greater and more rapid than are ordinarily met with in orchards, showed no relationship to either the occurrence or the severity of cracking.

Under natural orchard conditions the occurrence of cracking was always found to be associated with very low rates of evaporation. Rainfall was naturally confined to periods so characterized, but cracking was clearly correlated with the slow evaporation rather than with the rainfall. Thus, extensive cracking occurred in several instances of prolonged slow evaporation rates when there had been no rainfall for from 36 hours to 6 days; and in other instances no cracking was observed after heavy rains when the accompanying low evaporation rates were of short duration. An outbreak of cracking was generally preceded by a period of 6 hours or more during which a standardized black porous porcelain atmometer sphere with automatic recorder showed no measurable water loss and the water-saturation deficit of the air was very low. This correlation may be taken to indicate that outbreaks of cracking were generally brought about by greatly depressed transpiration, maintained for 6 hours or more. During a period of very slow transpiration the water supply available to the fruits should be very abundant, permitting unusually rapid enlargement of fruit cortex if other conditions are favorable for tissue swelling. If, at the same time, rain water is being absorbed appreciably through the peripheries of fruits and leaves, swelling of the fruit cortex should be still further increased.

Mercury manometers connected to attached branches where the branch tips had been cut off showed vascular suction at all times, even while nearby fruits were in process of cracking. No evidence was found to favor the supposition that excessive vascular pressure (commonly referred to as "root pressure") was generally present throughout these apple trees at any time; atmospheric pressure apparently always exceeded the hydrostatic pressure within the vessels of the branches. Attempts to produce cracking artificially by forcing water, under 5 to 10 pounds of excessive pressure per square inch, into the cut ends of detached branches bearing fruit failed when branch and fruit were exposed to the air of the laboratory and the treatment was continued for 3 hours or less.

Since change in soil-moisture content did not noticeably influence cracking in this study it appears that precipitation was not influential through wetting of the soil. Rainfall may be influential in other ways, however, for when fruit and leaf surfaces are more or less thoroughly covered with water during periods of rain, considerable water absorption may occur through those surfaces, while transpiration is correspondingly retarded or prevented. When rain was artificially diverted from large branches some of the apples on those branches cracked, although they and the accompanying leaves were thus kept superficially dry, which seems to indicate that the presence

of a film of water on foliage or fruit, or both, was not a necessary condition to promote cracking. On the other hand, when a fruit-bearing branch, still attached to the tree, was kept under water for several days the submerged apples showed severe cracking; and cracking was also artificially induced when detached apples, with stems and calyxes sealed, were kept under water for several days, suggesting that long-continued wetting of fruit and foliar surfaces by rain might aggravate the tendency to crack when other conditions tend to promote this injury. There is no doubt that the apples of this study were able to absorb water at a considerable rate when their skins were kept superficially wet. Enclosing attached apples in paper bags to prevent wetting of the skins by rain greatly reduced cracking in the apples so treated after the bags had been in place for 3 or 4 weeks; in that time, however, the internal conditions of the bagged apples may have changed greatly.

The occurrence and severity of cracking were not shown to be related in any way to air-temperature fluctuation.

Not all the apples on a branch or tree cracked when the weather conditions were favorable to cracking, and it is evident that some fruits were, at any time, more susceptible to cracking than others. A period of little or no evaporation apparently induced cracking of the currently most susceptible fruits only. With the recurrence of low evaporation rates additional fruits cracked, suggesting that this susceptibility of individual apples was likely to increase throughout the season. Some fruits cracked early in the season while others still remained sound at harvest time. A study of certain internal conditions that may have influenced predisposition to crack led to several apparently significant observations, as follows:

In a great majority of observed cases of cracking the injury was initiated in a region characterized by some form of visible abnormality or modification of the peripheral tissues of the fruit: viz, russeting, scab lesions, sunscald, or unusually high coloration. Such modifications usually were found on the side of the fruit that had experienced the greatest exposure to sun, wind, and spray injury. In russeted areas many minute cracks appeared to have been progressively formed and then closed by cork tissues, leaving the fruit periphery more or less roughened and mechanically weak in such regions.

Apparently the immediate mechanical cause of cracking is a form of tissue strain by which the skin and the adjacent tissues in a restricted area of the apple are excessively stretched because of enlargement in deeper lying tissues beneath the same area. With weather conditions suitable for crack formation, it seems that rupture occurs in the most susceptible peripheral areas, where the subperipheral pressure exceeds the limit of extensibility of the outer tissues. Thus the formation of a crack is determined partly by the mechanical strength of the peripheral tissue and partly by the magnitude of the pressure exerted by the enlarging tissue beneath.

It was observed that, among trees and branches otherwise apparently comparable, cracking was more pronounced and extensive when the foliage was sparse than when it was dense. This difference may have been related to the greater incidence of sunscald, russeting, and intense coloration in the fruits of trees and branches with poor foliage.

When attached apples were kept under water, cracking generally occurred first and most severely in those individuals that showed the most rapid rates of enlargement, which suggests that rapid enlargement of the entire fruit may tend to produce cracking when other conditions are suitable. But some slowly enlarging fruits cracked while some rapidly enlarging ones remained sound, and it seems that the excessive tissue swelling that resulted in a crack was sometimes effective only beneath the immediate peripheral region where rupture was about to occur.

Freezing-point measurements of osmotic values within the apple tissues showed, almost without exception, that tissue samples from regions beneath modified areas of the fruit periphery had significantly higher pressures than had similar samples from unmodified areas. For example, an apple with a russeted area on one side showed a high osmotic value beneath that area and a much lower osmotic value beneath the corresponding area on the opposite side of the fruit, with a gradient between these two regions. It therefore appears that the russeted or otherwise modified region was characterized not only by mechanical weakness of the periphery but also by exceptionally high tissue pressure in the deeper lying cortex. Both of these characteristics would be expected to favor cracking.

When aqueous dye solutions were fed into the cut ends of attached branches bearing fruit, the solutions were absorbed into the branches and carried into nearby apples, where the dyes became most concentrated in those parts of the peripheral regions characterized by russeting, scab lesions, or previously formed and more or less suberized cracks. Movement of vascular sap into the tissue beneath these modified regions and the passage of water out of them by transpiration were more rapid than elsewhere. With a low evaporation rate and consequent slow transpiration, it appears that the cortex beneath modified periphery may receive water more freely than other parts of the same fruit. This may promote excessive swelling beneath the modified areas.

LITERATURE CITED

(1) Baker, C. E.
1931. A study of skin structure of the Grimes apple as affected by different types of injury. Amer. Soc. Hort. Sci. Proc. (1930) 27: 75–81, illus.

(2) Boussingault, J.
1873. Sur la rupture de la pellicule des fruits exposés à une pluie continue. Endosmose des feuilles et des racines. Ann. Chim. et Phys. (4) 29: 360–367. [Abstract in Just's Bot. Jahresber. 1: 253. 1873.]

(3) Brooks, C., and Fisher, D. F.
1926. Some high-temperature effects in apples: contrasts in the two sides of an apple. Jour. Agr. Research 32: 1–16, illus.

(4) ——— and Fisher, D. F.
1926. Water-core of apples. Jour. Agr. Research 32: 223–260, illus.

(5) Burke, R. T. A., and McCall, H. F.
1919. Soil survey of Washington County, Maryland. U. S. Dept. Agr., Bur. Soils Adv. Sheets Field Oper. 1917, 46 pp., illus.

(6) Caldwell, J. S.
1928. Chemical composition of apple juices as affected by climatic conditions. Jour. Agr. Research 36: 289–365, illus.

(7) Campbell, J.
1928. Cracking of Dunn's and Cox's Orange apples. New Zeal. Jour. Agr. 37: 85–86.

(8) CHANDLER, W. H.
1925. FRUIT GROWING. 770 pp., illus. Boston, New York [etc.]
(9) COIT, J. E.
1917. CITRUS FRUITS, AN ACCOUNT OF THE CITRUS FRUIT INDUSTRY, WITH SPECIAL REFERENCE TO CALIFORNIA REQUIREMENTS AND PRACTICES AND SIMILAR CONDITIONS. 520 pp., illus. New York.
(10) DEVAUX, H.
1900. RECHERCHES SUR LES LENTICELLES; ÉTUDE SUR LES CONDITIONS PHYSIOLOGIQUES DE L'ACCROISSEMENT ET DE LA DIFFÉRENCIATION DE LA CELLULE ET DES TISSUS. Ann. Sci. Nat., Bot. (8) 12: 1–240, illus.
(11) FAWCETT, H. S., and LEE, H. A.
1926. CITRUS DISEASES AND THEIR CONTROL . . . 582 pp., illus. New York.
(12) GARDNER, V. R., BRADFORD, F. C., and HOOKER, H. D.
1922. THE FUNDAMENTALS OF FRUIT PRODUCTION. 686 pp., illus. New York.
(13) GOODWIN, B. C.
1929. BLISTER DISEASE OR CRACKING OF APPLES: SUCCESSFUL REMEDIAL MEASURES IN NELSON DISTRICT. New Zeal. Jour. Agr. 39: 305–307.
(14) GRAEBNER, P.
1920. LEHRBUCH DER NICHTPARASITÄREN PFLANZENKRANKHEITEN. 333 pp., illus. Berlin.
(15) HARRIS, J. A., and GORTNER, R. A.
1914. NOTES ON THE CALCULATION OF THE OSMOTIC PRESSURE OF EXPRESSED VEGETABLE SAPS FROM THE DEPRESSION OF THE FREEZING POINT, WITH A TABLE FOR THE VALUES OF P FOR $\triangle = 0.001°$ TO $\triangle = 2.999°$. Amer. Jour. Bot. 1: 75–78.
(16) HARTMAN, H., and BULLIS, D. E.
1929. INVESTIGATIONS RELATING TO THE HANDLING OF SWEET CHERRIES WITH SPECIAL REFERENCE TO CHEMICAL AND PHYSIOLOGICAL ACTIVITIES DURING RIPENING. Oreg. Agr. Expt. Sta. Bull. 247, 38 pp., illus.
(17) HEALD, F. D.
1933. MANUAL OF PLANT DISEASES. Ed. 2, 953 pp., illus. New York and London.
(18) LIVINGSTON, B. E.
1911. A RADIO-ATMOMETER FOR COMPARING LIGHT INTENSITIES. Plant World 14: 96–99.
(19) ———
1915. ATMOMETRY AND THE POROUS CUP ATMOMETER. Plant World 18: 21–30, 51–74, 95–111, 143–149, illus.
(20) RIXFORD, G. P.
1918. SMYRNA FIG CULTURE. U. S. Dept. Agr. Bull. 732, 43 pp., illus.
(21) SAWADA, E.
1931. STUDIES ON THE CRACKING OF CHERRIES. Agr. and Hort. 6: 865–892. [In Japanese, with English summary.]
(22) SCHILBERSZKY, K.
1918. HIPERTRÓFOS PARASZEMÖLESÖK ALMAGYÜMÖLOSÖKÖN. (HYPERTROPHE LENTIZELLEN AUF APFELFRÜCHTEN). Bot. Közlemenyek 17: 93. [Reviewed by Matouschek in Ztschr. Pflanzenkrank. 29: 249. 1919.]
(23) SORAUER, P.
1922. MANUAL OF PLANT DISEASES. Transl. by F. Dorrance. Ed. 3, v. 1, illus. [Wilkes-Barré, Pa.]
(24) SWINGLE, C. F.
1929. A PHYSIOLOGICAL STUDY OF ROOTING AND CALLUSING IN APPLE AND WILLOW. Jour. Agr. Research 39: 81–128, illus.
(25) TRACY, W. W.
1907. TOMATO CULTURE; A PRACTICAL TREATISE ON THE TOMATO, ITS HISTORY, CHARACTERISTICS, PLANTING, FERTILIZATION, CULTIVATION IN FIELD, GARDEN, AND GREENHOUSE, HARVESTING, PACKING, STORING, MARKETING, INSECT ENEMIES AND DISEASES, WITH METHODS OF CONTROL AND REMEDIES, ETC., ETC. 150 pp., illus. New York.

(26) VEIHMEYER, F. J.
1927. SOME FACTORS AFFECTING THE IRRIGATION REQUIREMENTS OF DECIDUOUS ORCHARDS. Hilgardia 2: [125]–290, illus.
(27) ———
1929. AN IMPROVED SOIL-SAMPLING TUBE. Soil Sci. 27: 147–152.
(28) VERNER, L.
1934. A SIMPLIFIED METHOD OF DETERMINING FREEZING-POINT DEPRESSIONS OF APPLE TISSUE WITH THE BECKMANN APPARATUS. Amer. Soc. Hort. Sci. Proc. 31: 33–34.
(29) ——— and BLODGETT, E. C.
1931. PHYSIOLOGICAL STUDIES OF THE CRACKING OF SWEET CHERRIES. Idaho Agr. Expt. Sta. Bull. 184, 15 pp., illus.

POTATO BEETLE SEPTICEMIA[1]

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INTRODUCTION

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), widely known as a pest of potato, also feeds on tomato, tobacco, eggplant, pepper, belladonna, henbane, and other solanaceous plants. It is, therefore, a pest of major importance. In an earlier note the writer [2] reported the existence of a bacterial disease of this insect. The present paper describes the disease and incorporates observations made on it from 1921 to 1929.

NAME OF THE DISEASE AND THE PATHOGENIC BACILLUS ASSOCIATED WITH IT

The potato beetle disease and the disease produced experimentally in insects inoculated by puncture with material from larvae sick or dead of the disorder are characterized by a marked septicemia. This suggested the name "potato beetle septicemia." The names "hornworm septicemia"[3] and "cutworm septicemia"[4] were coined earlier for similar diseases of hornworms and cutworms, respectively. A similar disease of grasshoppers, first described by D'Herelle [5], has received much attention.

The pathogen associated with the grasshopper disease is *Bacillus (coccobacillus) acridiorum* D'Herelle, the one associated with the hornworm disease is *B. sphingidis* White, and the one associated with the cutworm disorder is *B. noctuarum* White. In line with this method of choosing specific names for these organisms, the name *B. leptinotarsae* was given by the writer [6] to the pathogenic species associated with potato beetle septicemia. All these species are closely related and are members of a large group of organisms affecting insects which may be termed the "septicemia" group.

SYMPTOMS

Larvae affected by potato beetle septicemia are at first sluggish but soon become motionless. Their appetite is impaired, and they soon cease to feed. Usually they fall to the ground when moribund or dead, although dead ones are occasionally found adhering to the food plant (fig. 1, *A*). The appearance of sick larvae and of those recently dead is very similar to that of healthy ones. Soon after death the reddish tint of healthy larvae changes to a brownish gray. The

[1] Received for publication Apr. 23, 1935; issued September, 1935.

[2] WHITE, G. F. POTATO BEETLE SEPTICEMIA, WITH THE PROPOSAL OF A NEW SPECIES OF BACTERIUM. Ent. Soc. Wash. Proc. 30: 71–72. 1928.

[3] ——— HORNWORM SEPTICEMIA. Jour. Agr. Research 26: 477–486, illus. 1923.

[4] ——— CUTWORM SEPTICEMIA. Jour. Agr. Research 26: 487–496, illus. 1923.

[5] HERELLE, F. D'. SUR LA PROPAGATION, DANS LA RÉPUBLIQUE ARGENTINE, DE L'ÉPIZOOTIE DES SAUTERELLES DU MEXIQUE. Compt. Rend Acad. Sci. [Paris] 154: 623–625. 1912.

[6] WHITE, G. F. See footnote 2.

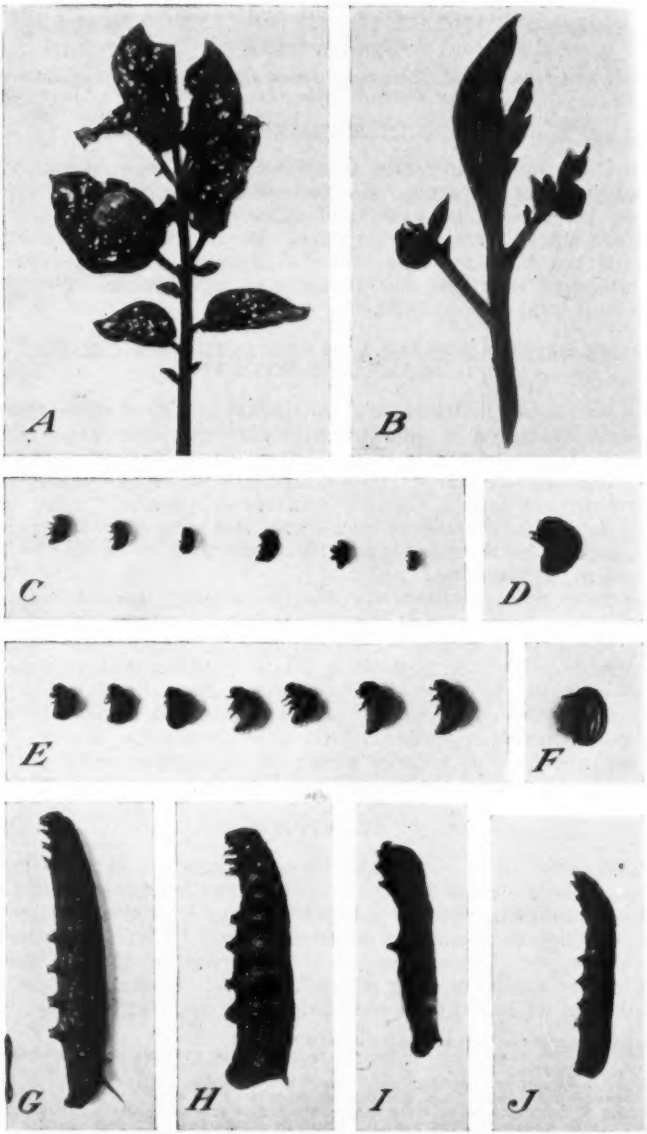


FIGURE 1.—Potato beetle septicemia: *A*, Larva sick of potato beetle septicemia, natural infection; *B*, larvae sick or dead of potato beetle septicemia, inoculated experimentally; *C* and *D*, larvae dead of potato beetle septicemia, natural infection. *E* and *F*, larvae and adult, respectively, dead of potato beetle septicemia, inoculated experimentally; *G*, catalpa sphinx larva inoculated with *Bacillus leptinotarsae*; *H*, hornworm inoculated with *B. leptinotarsae*; *I*, silkworm inoculated with *B. leptinotarsae*; *J*, cutworm inoculated with *B. leptinotarsae*.

black markings remain unchanged. As decay advances the color deepens, becoming a dark gray, dark brown, or nearly black.

The dead larvae retain for a while the surface contour of healthy ones; but as drying takes place, the surface of the body becomes roughened, and within a week the remains are a shriveled, dark, brittle mass (fig. 1, *C*, *D*). During most of the period of decay the resistance of the body wall to tearing is similar to that of healthy larvae. The decaying mass is nonviscid. Usually the odor is slight and not particularly disagreeable.

TECHNIC

The laboratory methods used in these studies were in general similar to those commonly employed in disease work. Good results were obtained from the incubation of cultures at room temperature or at approximately 30° C. In addition to the usual laboratory animals, larvae and adults of the potato beetle, silkworms (*Bombyx mori* L.), hornworms (*Phlegethontius quinquemaculata* Haw. and *P. sexta* Joh.), various species of cutworms and white grubs, and caterpillars of the catalpa sphinx (*Ceratomia catalpae* Bdv.) were used for experimental purposes. The puncture and feeding methods employed in earlier studies [7] were followed. When a fine needle was used and care was exercised to limit the injury caused to the body wall, death resulted only occasionally from trauma inflicted by puncture inoculations.

Potato plants growing in pots served well as food for the larvae and adult potato beetles after they had been inoculated. Following puncture inoculations the insects were placed immediately on the potted plants. In the feeding inoculations the plants were sprayed with an aqueous suspension of disease material or with cultures, and the insects to be inoculated were placed on them while the plants were still wet. In inoculations by immersion the insects were immersed in a beaker containing the culture and afterward placed on a growing potato plant.

A cardboard or other suitable paper was fitted about the base of the plant for collecting dead or sick insects as they fell from the plant or otherwise left it. The mature, unaffected larvae leaving the plant may escape and, reaching the soil within the pot, burrow into it for pupation. Moribund larvae and even dead ones occasionally are found adhering to the plant (fig. 1, *B*), as was observed in potato beetle septicemia encountered in nature.

POST-MORTEM CHANGES IN EXPERIMENTAL INSECTS

The changes occurring in the bodies of insects that die from experimental infection with cultures of *Bacillus leptinotarsae* vary somewhat with the different insect species used, and slightly with the different cultures employed in the inoculations. Temperature and moisture tend to modify the speed of the changes that occur and to some extent their character. Potato beetle larvae (fig. 1, *E*) that died from experimental inoculation showed post-mortem changes similar to those observed in larvae that died of the disease in nature.

[7] WHITE, G. F. See footnote 3.

Silkworms (fig. 1, *I*) in the fifth instar inoculated with disease material of infected insects or with pure cultures of *Bacillus leptinotarsae* die within 24 hours when the temperature of the room is 30° C. or higher. Soon after death the remains turn a light brown, which is preceded at times by a slightly greenish hue. At first the color changes are not uniform over the entire body but by the end of 24 hours they become so. The hue is then brown which gradually darkens until it becomes almost black. The body wall soon yields easily to tearing, and the contents become a moist, thick, brown mass. When allowed to dry the remains become brittle by the end of a week. The odor from the bodies is not particularly offensive when they are left in the open, but becomes so when they are confined.

The post-mortem changes in hornworm larvae (fig. 1, *H*) that have died from experimental infection with *Bacillus leptinotarsae* are similar to those noted for silkworms.

The bodies of catalpa sphinx larvae (fig. 1, *G*) that have died from experimental inoculation with the bacillus soon turn brown. During the first day of post-mortem changes the body wall will usually support the weight of the dead larva, but later it becomes less resistant.

The bodies of cutworms (fig. 1, *J*) dead from experimental infection become a deep brown within 2 days. The body wall is then easily torn, and the decaying tissues are a moist, thick, brown mass which is brittle after it becomes dry.

White grubs that have died from puncture inoculations may first present a mottled appearance, but later they become a brown which may appear almost black.

OCCURRENCE OF POTATO BEETLE SEPTICEMIA

A disease of the septicemia group may be suspected when cultures made from insects dying in nature yield a luxuriant growth of numerous colonies of a small actively motile bacillus. Such a disease is further indicated when silkworms or other experimental insects inoculated by puncture with the bacillus die within 1, 2, or 3 days and death is accompanied by a septicemia and followed by post-mortem changes characterized by a dark-brown color and softening of the remains.

On June 10, 1921, Doris H. Blake, of the Bureau of Entomology, while engaged in biological studies on the potato beetle, found larvae in Washington, D. C., that were dead from a cause unknown to her. Two silkworms inoculated by puncture with an aqueous suspension of one of these larvae died within 24 hours. Five silkworms inoculated with the blood from the two dead silkworms died also. The blood of these recently dead worms contained many short motile rods. Agar plates made from the bodies yielded numerous gray colonies of an actively motile bacillus. The bodies became soft and turned brown. The presence of a disease of the septicemia group was therefore indicated.

On August 29, 1921, W. H. White, also of the Bureau of Entomology, collected sick potato beetle larvae from potato plants at Arlington, Va. A short, actively motile bacillus that produced a gray growth was isolated from each of the larvae that were cultured. Two healthy potato beetle larvae were inoculated with cultures from

each sick larva. All the inoculated larvae died within 2 days. The indications, therefore, were that this disease also belonged to the septicemia group.

On August 15, 1922, the writer collected about 50 potato beetle larvae, some sick and others recently dead, from potato plants grown on the plot in Washington where Blake had found diseased larvae the year before. Some of the larvae were adhering to the food plants, although most of them were on the surface of the ground about the plants. Twelve of these were cultured, and each yielded gray colonies of an actively motile bacillus. A culture was made from a gray colony from each of the 12 plates. Inoculation of silkworms with these cultures showed 11 to be pathogenic, indicating a disease of the septicemia group. It seemed important that the culture from one gray colony was not pathogenic.

On August 20, 1923, a search was made for potato beetle larvae on the potato plants of the plot in Washington where, during each of the 2 preceding years, diseased larvae had been collected. Only six small to medium-sized larvae were found. Three of these were dead, but the other three presented no noticeable symptoms of disease. Agar plates made from each of the six larvae yielded gray colonies in each instance. Five silkworms were inoculated from each plate, a different colony being used for each worm. All the 30 worms died on the following day, the remains in each instance soon becoming soft and brown. These observations indicated that the 3 dead larvae had died of potato beetle septicemia and that the other 3, while not yet showing symptoms of the disease, were infected.

No dead potato beetle larvae were found during 1924 on the potato plot where during each of the 3 preceding years the disease had been encountered. Death from the disease may have occurred, however, since dead larvae may easily pass unnoticed on soil which has recently been cultivated and which has not been smoothed and hardened by rains.

The potato plot in Washington which offered the opportunity for a study of the occurrence of potato beetle septicemia and furnished much of the material for these studies had been used for potatoes for a number of years. As the primary purpose in maintaining the plot was to provide material for biological studies on the Colorado potato beetle, at least two plantings were made in it each year. After 1924 the plot was used for another purpose.

VIRULENCE OF BACILLUS LEPTINOTARSAE

Other data regarding the virulence of *Bacillus leptinotarsae* are shown in table 1, which gives the results with 15 cultures, each isolated from a different larva found in nature sick or dead of potato beetle septicemia.

The results recorded in table 1 may be summarized as follows: Of 88 potato beetle larvae inoculated, 85 died within 2 days; of 244 silkworm larvae, 243 died within 1 day; all of 15 hornworm larvae died within 2 days; all of 17 cutworm larvae died within 3 days; all of 19 catalpa sphinx larvae died within 2 days; all of 4 white grubs died within 2 days; and of 19 adult potato beetles inoculated, 15 died within 11 days.

TABLE 1.—*Tests showing the virulence of Bacillus leptinotaraae to various insects*

Year	Potato beetle larvae				Silkworm larvae				Hornworm larvae				Cutworm larvae				Catalpa sphinx larvae				White grubs				Adult potato beetles			
	Cultures tested	Larvae inoculated	Larvae dead	Period, inoculation to death	Cultures tested	Larvae inoculated	Larvae dead	Period, inoculation to death	Cultures tested	Larvae inoculated	Larvae dead	Period, inoculation to death	Cultures tested	Larvae inoculated	Larvae dead	Period, inoculation to death	Cultures tested	Larvae inoculated	Larvae dead	Period, inoculation to death	Cultures tested	Grubs inoculated	Grubs dead	Period, inoculation to death	Cultures tested	Adults inoculated	Adults dead	Period, inoculation to death
	No.	*No.*	*No.*	*Days*	*No.*	*No.*	*No.*	*Days*	*No.*	*No.*	*No.*	*Days*	*No.*	*No.*	*No.*	*Days*	*No.*	*No.*	*No.*	*Days*	*No.*	*No.*	*No.*	*Days*	*No.*	*No.*	*No.*	*Days*
1921	4	11	11	2	2	10	10	1	4	15	15	2	2	2	2	3												
1922	6	11	18	2																								
1923	5	18	37	2		139	139	1						15	15	3		19	19	2		4	4	2	4	19	15	11
1924					7	35	35	1					4	15			1	4										
1925					5	24	24	1																				
1926	5	20	19	2	7	26	26	1																				
1928					3	9	9	1																				
1929																												

It will be observed that among the larvae inoculated by puncture with *Bacillus leptinotarsae* an extremely high mortality resulted within 3 days. Adults are not so susceptible to infection with this organism. It will be noted further that the virulence of cultures on artificial media is retained over long periods.

Feeding and immersion methods were also employed in attempts to transmit the disease to potato beetle larvae. In two tests in which 42 larvae were inoculated by feeding, 7 died within 8 days.[8] Fifty larvae were placed on potted potato plants which had been sprayed with a 48-hour bouillon culture of the bacillus. No evidence of infection was seen among these. Of 75 larvae immersed in an aqueous suspension of an agar culture and then placed on a potted potato plant, 3 died within 5 days. Whether these 3 died from the inoculation is not known.

EXCITING CAUSE

Bacillus leptinotarsae is the bacillus found in potato beetle septicemia in nature and the one present in the septicemia experimentally produced in the insects inoculated by puncture with material from the

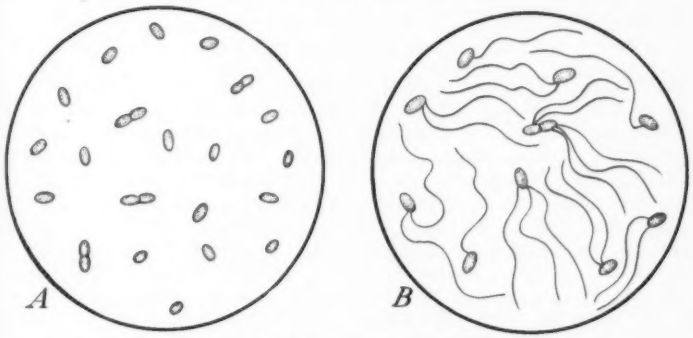


FIGURE 2.—*A, Bacillus leptinotarsae; B,* flagella of *B. leptinotarsae.*

potato beetle disease. Both in the disease as it occurs in nature and in the experimental one this bacillus seems to be the immediate cause of death. Whether it is the primary exciting cause of the potato beetle disorder is yet to be determined.

Inoculation of the common and usual differential media with *Bacillus leptinotarsae* is followed by abundant growth.

MORPHOLOGY.—The common name "coccobacillus" suggests the morphology of the species. The rods from a 1-day agar culture are small and short with rounded ends (fig. 2, *A*). Some are ovoid, and many resemble cocci. They measure about 0.9μ in length and 0.6μ in width. The rods from a 1-day bouillon culture measure 1.1μ in length. In older cultures they average slightly longer. Flagella are peritrichic (fig. 2, *B*); frequently only 1 is seen, and it is not polar. More often there are 2, 3, or 4, but rarely more than 4. Spores are not produced.

MOTILITY.—Many of the shorter rods progress actively.

STAINING PROPERTIES.—The rods stain readily and uniformly. They are Gram-negative.

AGAR PLATES.—Growth takes place rapidly. The colonies are gray by reflected and bluish by transmitted light. The border is entire and the surface oval and

[8] Three of the 7 dead larvae were parasitized by fly larvae. The gross appearance of the others did not indicate that they had died from infection with *B. leptinotarsae.*

glistening. Magnified, they are finely granular. The mass is nonviscid and adheres to the medium.

AGAR SLANT.—Within 1 day a moderate bluish-gray nonviscid growth takes place.

GELATIN STAB.—A white growth with beginning of liquefaction is present along the stab after 24 hours at room temperature. The liquefaction continues and becomes crateriform, infundibuliform, and sometimes stratiform in 5 days. A friable pellicle forms which later sinks.

POTATO.—A moderate moist gray growth occurs within 2 days, which may become slightly brownish.

BOUILLON.—Growth takes place rapidly, the medium becoming slightly clouded within a few hours and heavily clouded within a day. A ring of gray growth adheres to the wall of the tube. A friable pellicle may form and later sink. The medium remains clouded and the sediment becomes heavy.

MILK.—A soft coagulum forms after 2 or 3 days and slow digestion takes place.

LITMUS MILK.—The milk is unchanged during the first day. Within a week the color is discharged.

CARBOHYDRATES.—Growth is usually increased by the addition of carbohydrates. Fermentation with acid production takes place in dextrose, galactose, levulose, maltose, mannose, mannite, glycerin, dextrin, xylose, and the glucoside salicin. The slight acidity occurring in lactose and arabinose is soon changed to alkaline. No acid is formed in raffinose, inulin, erythrite, or inosite.

THERMAL DEATH POINT.—A suspension in physiological salt solution is killed within 10 minutes at 52° C.

RESISTANCE TO DRYING.—An aqueous suspension from a 4-day agar culture on drying is dead within 3 hours.

PATHOGENESIS

Practically 100 percent of the potato beetle larvae inoculated by puncture with pure cultures of *Bacillus leptinotarsae* die from the infection produced. The period from inoculation to death ranges from less than 1 to more than 3 days. The course of the experimental disease varies with the insect species inoculated, the temperature, and slightly with the culture employed. Some cultures kept on agar with occasional transfers have retained their virulence for silkworms for more than 8 years. That of others has diminished.

A white mouse receiving intraperitoneally about 100,000 bacilli from a 48-hour agar culture became ill within 2 hours, later lying on its belly with the flanks depressed. Within a few hours it recovered and remained well thereafter. A rabbit similarly inoculated with about 200,000 bacilli was somewhat sluggish 4 hours after the inoculation, but soon recovered and remained well. A guinea pig, white rat, and two chickens receiving intraperitoneally about 200,000 bacilli manifested no symptoms of disease.

The recovery of *Bacillus leptinotarsae* from potato beetle larvae sick or recently dead of potato beetle septicemia, the experimental infection of healthy potato beetle larvae with a pure culture of the species by the puncture method, followed by death of the larvae with post-mortem changes similar to those of potato beetle septicemia, and the recovery of the bacillus in pure culture from the tissues of the experimental insect are easily accomplished. These observations tend strongly to indicate that the bacillus is the inciting cause of potato beetle septicemia. However, the extremely low mortality that follows both the feeding and the immersion methods of inoculation lead one to inquire further regarding the primary exciting cause of the disorder.

CULTURES FROM APPARENTLY HEALTHY LARVAE

Examinations were made of apparently healthy potato beetle larvae to determine the presence or absence of pathogenic bacterial species in the intestines. A bit of feces expressed from a larva and streaked on the surface of an agar plate usually sufficed for obtaining fairly well isolated colonies. One plate was made from each larva examined. Silkworms were inoculated by puncture from these plates, each worm receiving a culture from a different colony. Ordinarily five worms were inoculated from each plate. When the inoculated worms manifested symptoms and died, and the postmortem changes resembled those of worms that had died from inoculation with *Bacillus leptinotarsae*, this pathogenic species was suspected.

The apparently healthy larvae, studied during 1923, were taken from potato plants grown on the same plot of ground where larvae, dead of potato beetle septicemia, had been found during each of the 2 preceding years. Gray colonies resembling those of *Bacillus leptinotarsae* were selected for examination.

On June 8, silkworms were inoculated from 3 plates. None of the 15 worms inoculated from these died. On June 9, silkworms were inoculated from 7 other plates. All the 25 worms inoculated from 5 of these plates remained well. All of the 10 worms inoculated from the other 2 plates died. On June 11, silkworms were inoculated from 12 plates. None of the 55 larvae inoculated from 11 of these plates died. Two of the five worms inoculated from the other plate died, the remaining 3 being unaffected.

The other observations for 1923 on apparently healthy potato beetle larvae are as follows: All the 10 gray colonies examined from 2 of 35 larvae were pathogenic to silkworms; 3 of the 5 colonies examined from another larva were pathogenic; and none of the colonies selected from plates made from the remaining 32 larvae were pathognic.

The bacterial species isolated from apparently healthy potato beetle larvae which formed gray colonies and which was pathogenic to silkworms was found upon cultural study to be *Bacillus leptinotarsae*, the others were not.

Further studies were made during 1924 on the bacteria of apparently healthy potato beetle larvae. A species producing yellow colonies, one producing gray colonies similar to those of *Bacillus leptinotarsae*, and a species of *Streptococcus* were examined in addition to *Bacillus leptinotarsae*. Most of the larvae studied were taken from the same potato plot from which the larvae examined in 1923 had been collected.

On June 18, 10 agar plates were made, each with a bit of feces from a different potato beetle larva, and the gray colonies were chosen for testing. All of the 20 worms inoculated from 4 of the plates died, 14 of the 20 worms inoculated from 4 other plates died, and all of the 10 worms inoculated from the remaining 2 plates lived.

On July 15, 11 agar plates were made from 11 apparently healthy potato beetle larvae obtained from a locality 8 miles distant from the plot in Washington which had furnished larvae for the studies reported above. When 4 gray colonies from each plate were used, none of the 44 silkworms inoculated from the 11 plates died.

On June 25, 14 agar plates were made from 14 healthy larvae. In each instance the expressed feces were added to 1 cc of sterile water, and a plate was made from the suspension. Gray colonies were obtained on 10 of the 14 plates, yellow ones on 12 of them, and a species of *Streptococcus* on 6 of them. The number of yellow colonies per plate ranged from 1 to 14; the gray ones, from 1 to 300; and the *Streptococcus*, from 2 to 200, the larger counts being estimated in each instance.

On September 2, 16 agar plates were inoculated each with the diluted feces from a different potato beetle larva. One of the plates remained sterile. The rest bore gray colonies ranging in number from a few to many. Some of them also contained yellow colonies of a nonpathogenic, nonmotile bacillus and a few contained a species of *Streptococcus*. The pathogenic species forming gray colonies studied during 1924 were found to have the cultural characteristics of *Bacillus leptinotarsae*.

From this preliminary study on apparently healthy potato beetle larvae it is seen that some larvae taken from a plot where potato beetle septicemia had occurred harbored *Bacillus leptinotarsae*, and that a nonpathogenic bacillus resembling *B. leptinotarsae*, a nonpathogenic streptococcus, and a nonmotile, nonpathogenic chromogen were often present also.

COMPARISON OF BACILLUS LEPTINOTARSAE, B. SPHINGIDIS, AND B. NOCTUARUM

The morphology and cultural characteristics of *Bacillus leptinotarsae*, *B. sphingidis*, and *B. noctuarum* are similar. Variations within each species are comparable to the slight variations noted for the different species. The pathogenesis of these different species observed when silkworms are inoculated by puncture is also similar. Differences have been found in agglutination tests for the different species. Agglutination variations, too, are found among the different cultures of *B. leptinotarsae* isolated from the same outbreak of the disease. Serum from a rabbit immunized with *B. leptinotarsae* showing an agglutination titer of 1 : 1,600 for the culture used agglutinated some other cultures of the species at 1 : 1,600, some at 1 : 400, some at 1 : 200, and some were not agglutined.

In giving another name to the bacillus occurring in potato beetle septicemia which is so similar to the species occurring in hornworm septicemia and to the one in cutworm septicemia, the writer is following the example of others working on this disease group. When the group has been more fully studied a different classification of these species may be made.

DIAGNOSIS OF POTATO BEETLE SEPTICEMIA

The presence of potato beetle septicemia may be suspected if the number of larvae in a beetle infestation should diminish rapidly from no apparent cause. The disease is further indicated if dead larvae of different ages showing post-mortem changes characteristic of the disease are found on the ground. A positive diagnosis is made by finding *Bacillus leptinotarsae* in sick or dead larvae. The disease must be differentiated from insect parasitization and death from insecticides.

PREDISPOSING CAUSES OF THE DISEASE

If *Bacillus leptinotarsae* is the primary exciting cause of potato beetle septicemia, predisposing factors probably play an important role in the causation of the disease. Since few if any potato beetle larvae died from feeding or immersion inoculations, these insects, it seems, must possess protective agencies which tend to prevent the entry of the organism into the blood.

Most of the larvae found dead of the disease in nature were either small or of medium size. In experimental inoculations also larvae of these sizes died sooner than those that were nearly mature.

Death from inoculations takes place sooner during hot weather than when the temperature is lower. That the season of the year is a predisposing factor for the disease is indicated by the observations that the heaviest mortality occurs in August.

TRANSMISSION OF THE DISEASE

Since the intestinal tract, the blood, and various tissues of the potato beetle larvae are places of multiplication of *Bacillus leptinotarsae*, the feces of such larvae and the bodies of those dead of the disease may contaminate the soil with the bacillus. As the viability of the bacillus is well maintained in damp earth, the soil remains a possible source of infection for long periods.

While it seems likely that the bacillus may at times gain entrance to the blood of the larvae by way of the alimentary tract, experimental evidence indicates that the insect possesses protective forces which markedly restrict infection by this route. Owing to the ease with which infection may be induced by puncture inoculations, injuries to the body wall by enemies of the larvae are to be considered as possible factors in the transmission of the disease. Among enemies reported for the potato beetle are a ladybird, ground beetles, soldier bugs, robber flies, a tachinid fly, mites, and spiders.

BACILLUS LEPTINOTARSAE AS A FACTOR IN THE CONTROL OF THE COLORADO POTATO BEETLE

In each year of the three that potato beetle septicemia was found in the field the presence of the disease was accompanied and followed by a rapid decrease in the number of larvae in the infestation, although there were at the time an abundance of food and many adult beetles. In the absence of any known cause for the decrease the disease must be considered a possible causative factor.

The heaviest mortality accompanying the disease has been noted among larvae of the last brood for the year. If the overwintering insects should be largely of this brood the disease would seem to have an added importance in the natural control of the pest.

Inasmuch as the disease is not readily transmitted through feeding inoculations with *Bacillus leptinotarsae*, the spraying of potato plants with cultures of this organism cannot be recommended at the present time as a means of artificial control. Whether artificial contamination of the soil with disease material or some other artificial use of the disease would be helpful in effecting control are problems for further study.

More data on the distribution and seasonal incidence of, and mortality from, the disease are much needed. It would be particularly instructive to know what insect and other enemies of the potato beetle are operating before and during the occurrence of the disease. Such observations would furnish useful information regarding the causes of the disease, its importance in the natural control of the pest, and its possible use in artificial control measures.

SUMMARY AND CONCLUSIONS

The present paper includes a description of, and a report on, preliminary studies of a disease of the Colorado potato beetle.

A frank septicemia in the moribund insect is a marked characteristic of the infection. This fact suggested the name potato beetle septicemia for the disease.

The bacillus occurring in the septicemia is a short, actively motile, Gram-negative rod, to which has been given the name *Bacillus leptinotarsae*.

Another bacillus found in cultures from larvae dead of potato beetle septicemia is morphologically and culturally similar to *B. leptinotarsae*, but is not pathogenic.

A nonpathogenic streptococcus occurred in considerable numbers in some of the larvae sick or dead of the disease.

Plate cultures made from feces expressed from healthy potato beetle larvae usually yielded only a small number of colonies. Only a few species occurred, and these often were chromogens.

The virulence of cultures of *B. leptinotarsae* on artificial media has changed little in 8 years.

It seems probable that potato beetle septicemia is a factor in the natural control of the Colorado potato beetle. Further studies are needed to demonstrate its importance in artificial control measures.

ROSE BLAST INDUCED BY PHYTOMONAS SYRINGAE [1]

By H. R. Rosen

Plant pathologist, Arkansas Agricultural Experiment Station

INTRODUCTION

Early in May 1933, a malady which apparently has not been described was detected in a rose garden at Fayetteville, Ark., on the well-known hybrid perpetual, Magna Charta. It involved receptacles, calyx lobes, flower stalks, and leaf petioles, being most abundant on receptacles and relatively rare on leaf petioles.

SYMPTOMS

The affected portions appeared as blackish-brown, dead spots and streaks, which varied considerably in size and shape (fig. 1). Some took the form of small oval-shaped or rounded spots, not more than 1 mm in diameter, while others were large narrow streaks involving 5 to 8 cm of tissue in the longest diameter. The individual spots and streaks appeared depressed or sunken as compared with adjoining healthy tissue, particularly on the receptacles and pedicels, and suggested a collapse of the cells involved in the diseased areas. A narrow red border often delimited the diseased parts. On the large calyx lobes, characteristic of Magna Charta, the disease, starting at the base, often ran the whole length of the lobe. In most instances the affected buds failed to open, and may be said to have been blasted or blighted.

Microscopic examinations of diseased areas revealed great numbers of bacteria within the tissues, the bacteria readily clouding the water medium when slight pressure was applied to the cover glass. In this respect the disease closely resembed fire blight on rose buds. Indeed, the external symptoms bear a very close resemblance to those pictured by Rosen and Groves [2] on rose buds artificially infected with *Erwinia amylovora*, except that droplets of bacterial exudate were not observed in the disease here discussed.

INOCULATION EXPERIMENTS

Several pure-culture isolations were attempted, and isolates were obtained from two diseased receptacles, a calyx lobe and a petiole. Special studies were made of the morphology, the cultural and physiological reactions, and the pathogenicity of these isolates. Since artificial inoculations on shoots of pear (*Pyrus communis*) fig. 2) resulted in blackish discoloration of leaf blades that were

[1] Received for publication Mar. 27, 1935; issued September 1935. Research Paper No. 391, Journal Series, University of Kansas.

[2] Rosen, H. R., and Groves, A. B. studies on fireblight: host range. Jour. Agr. Research 37: 493–505, illus. 1928.

located considerably beyond the bacteria-invaded stem tissue, it appeared that the pathogen might belong to the *Phytomonas syringae* group and not to *Erwinia amylovora* (see Rosen and Bleeker).[3] To test this theory the rose isolates were grown on Uschinsky's solution and on beef-infusion agar, two media which are conducive to the

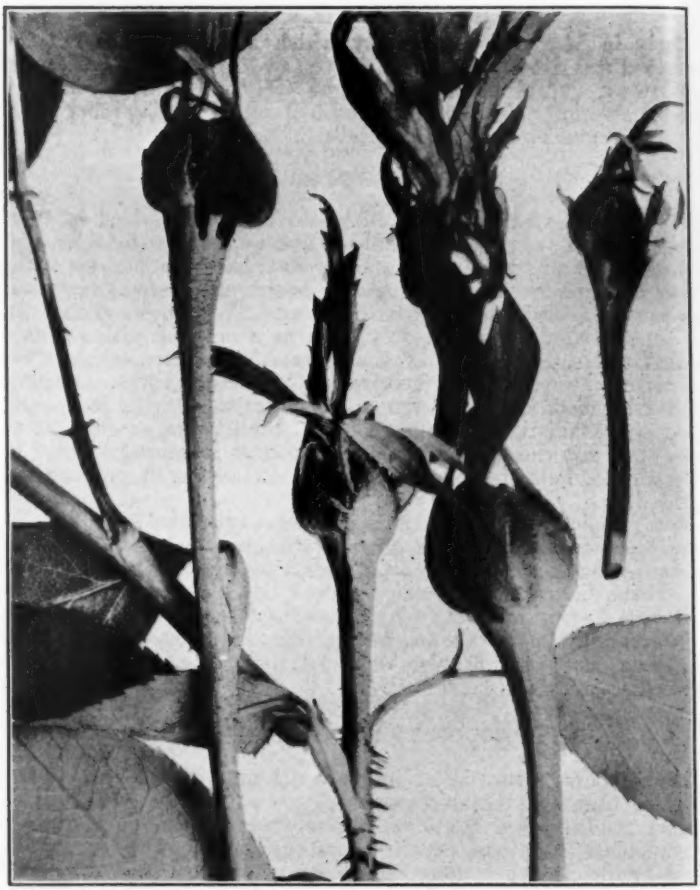


FIGURE 1.—Natural infections on rose calyx lobes, receptables, pedicels, and petiole (extreme left) of Magna Charta. Slightly reduced.

development of the greenish fluorescent pigment characteristic of *P. syringae*. All of the rose isolates, when transferred to these media, developed a pigment which appeared very similar to that produced by authentic cultures of *P. syringae*, *P. citriputeale*, and *P. prunicola*.

[3] ROSEN, H. R., and BLEECKER, W. L. COMPARATIVE SEROLOGICAL AND PATHOLOGICAL INVESTIGATIONS OF THE FIRE-BLIGHT ORGANISM AND A PATHOGENIC FLUORESCENT GROUP OF BACTERIA. Jour. Agr. Research 46: 95–119, illus. 1933.

Former work by Rosen and Bleecker had shown the lemon to be a differential host between *Erwinia amylovora* and *Phytomonas syringae*, the fire blight pathogen producing little or no effect on this fruit while the various forms of *P. syringae* produced typical black pit lesions. Consequently, if the rose isolates were related to *P. syringae*, they should produce conspicuous blackish depressed spots on lemons.

FIGURE 2.—Artificial infections on Bartlett pear shoots with a pure-culture isolate from a diseased rose receptacle. Note blackish discolorations of stem tissues (around points of inoculation), and the sootylike discolorations of the extreme upper leaf blade, central shoot, characteristic of such artificial infections on pear, and serving to distinguish *Phytomonas syringae* from *Erwinia amylovora*.

Needle-prick inoculations on 12 lemons with these rose pathogens, contrasted with *E. amylovora* inoculations made at the same time, resulted in the production of blackish spots with the rose isolates and none with the fire blight pathogen (fig. 3). The spots were characteristic of those produced by *P. citriputeale* (one of the many names under which *P. syringae* has been described). For a relatively rapid

and simple differential test between *E. amylovora* and fluorescent rosaceous bacterial pathogens, the use of lemon fruit appears to offer exceptional advantages.

In addition to pear twigs and lemon fruit, artificial inoculations were made on two varieties of roses, Magna Charta and Radiance (pink), and on young lilac shoots. All the inoculations on Magna Charta were made out of doors in a garden which had shown no

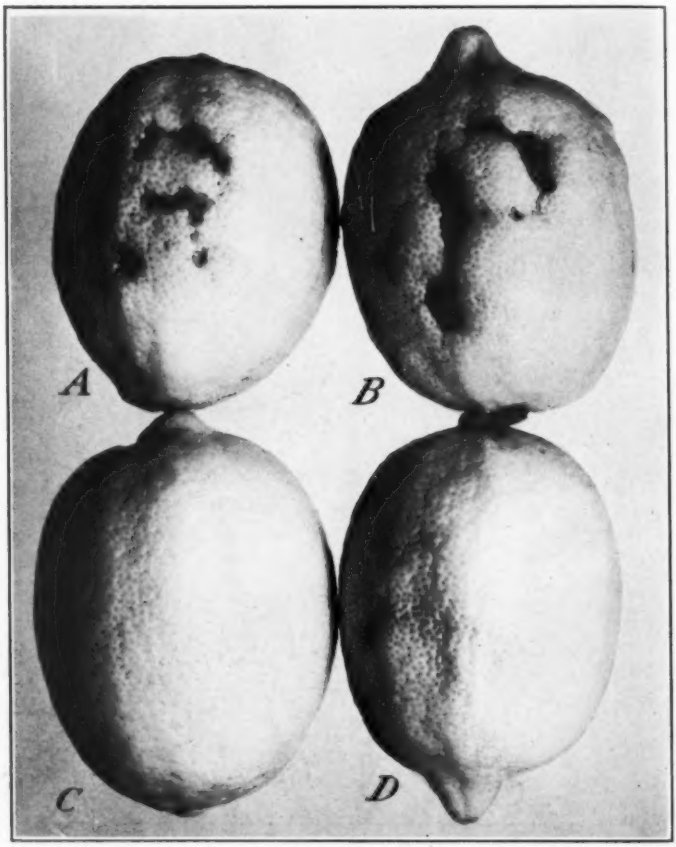


FIGURE 3.—Artificial needle-puncture inoculations with pure-culture isolates from a diseased rose receptacle (*A*), and a diseased leaf petiole (*B*),; control, needle-puncture injections with sterile water (*C*), and with *Erwinia amylovora* (*D*). Note marked infections with the rose isolates, and the little or no effect of *E. amylovora*.

signs of the disease. On April 29, 1934, 11 flower buds of this variety were inoculated by means of needle punctures comparable to wounds made by thorn injuries, the inoculum consisting of 4-day-old nutrient dextrose-agar slant cultures of a rose-receptacle isolate suspended in sterile distilled water. Several leaf and flower clusters were atomized with similar water suspensions without wounding, the

bacteria being applied by means of sterilized, all-metallic artists' atomizers. These atomizer inoculations were enclosed in glassine bags. A similar number of control inoculations were made by means of needle punctures and the use of sterile water, and by atomizing unwounded shoots with sterile water and covering them with glassine bags. On May 3 all needle-puncture inoculations with bacteria showed symptoms resembling natural infections of rose blast (fig. 4). All the controls were free from disease symptoms other than very limited brownish spots centering around the needle pricks. By May 8 the infected areas on some of the buds inoculated by needle punctures had involved one-third or more of the receptacle circumference, 1 or 2 calyx lobes, and the upper third of the pedicels. In these inoculations, the portion of the bud which showed no depressed, brownish areas turned a sickly yellow and the buds with attached pedicels aborted and dropped from the plant. All checks remained healthy, the blossoms opening normally and remaining attached to the plant. No sure signs of infection were obtained in the unwounded atomizer inoculations. From one of the artificially infected buds the organism was reisolated, grown in pure cultures, and inoculated on several Bartlett pear shoots, on which typical *Phytomonas syringae* symptoms appeared.

Numerous attempts to infect Radiance plants maintained in a greenhouse by needle-puncture inoculations, by hypodermic injections, and by spraying with bacterial water suspensions on young buds and leaves, were failures. Beyond wound injuries made by the needles, the inoculated plants showed no signs of disease. Under greenhouse conditions at least, Radiance appears to be nonsusceptible to this disease when Bartlett pear shoots similarly inoculated are very susceptible.

On April 5, 1934, tender shoots of lilac (*Syringa vulgaris*) attached to plants growing out of doors were inoculated with different isolates of *Phytomonas syringae* by means of hypodermic injections. The first signs of infection appeared on April 9. Ten shoots were inoculated with each isolate. The number infected is shown below.

Isolate:	*Number of shoots infected*
Rose receptacle	8
Pear receptacle [4]	7
Pear petal [4]	7
P. syringae from lilac [4]	1
P. prunicola [4]	0
Pear (Garber) [4]	0
Controls (sterile water)	0

In addition to the hypodermic injections, several lilac leaf clusters were inoculated without wounding by atomizing with water suspensions of the following isolates: Rose receptacle, pear petal, and *Phytomonas syringae*. Six days later the only lilac leaves infected were those atomized with the isolate from rose receptacle, and even these were very small, pin-point lesions on a few leaves. These results coupled with those shown above, leave no doubt that the rose isolate is parasitic on lilac (fig. 5). The fact that the rose isolate

[4] These isolates were the ones previously studied by Rosen and Bleecker. See footnote 3.

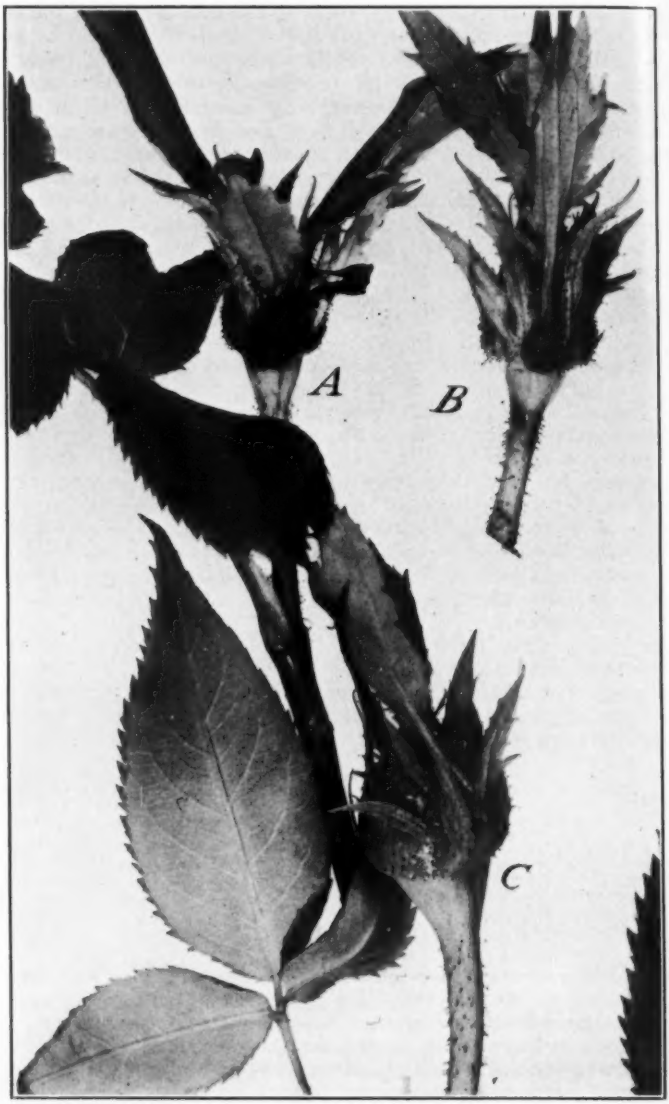


FIGURE 4.—(*A*, *B*), Artificial needle-puncture inoculations on Magna Charta rose with a pure-culture isolate from rose blast; *C*, needle puncture on control. Photographed 9 days after inoculating. Compare with figure 1.

produced a greater number of infections on this host than the lilac blight isolate can perhaps be explained by the fact that the latter had been kept growing in artificial cultures for several years and had

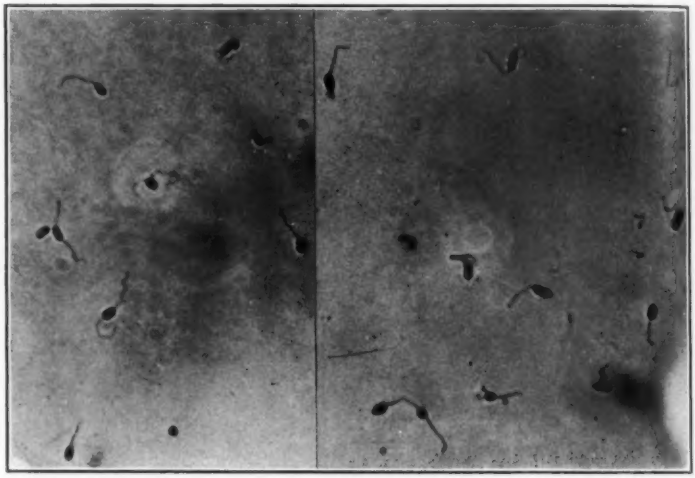
FIGURE 5.—Artificial infections on lilac with a pure-culture isolate from rose blast. Photographed 5 days after inoculating.

probably lost most of its virulence. The same explanation may be offered for lack of infections with several of the other isolates. The stem lesions produced by the rose blast isolates on lilac are comparable to those described by Bryan [5] for lilac blight.

[5] BRYAN, M. K. LILAC BLIGHT IN THE UNITED STATES. Jour. Agr. Research 36: 225-235, illus. 1928

CULTURAL CHARACTERS OF THE ORGANISM

In culture media and in stained preparations, the rose blast isolates are comparable to the other isolates noted previously. They are all rapidly growing, motile, polar-flagellated rods (fig. 6), which as isolated colonies on nutrient agar plates are round, glistening, white or grayish white, butyrous, pulvinate, and definite-margined. If beef-infusion agar is used instead of beef extract, the agar around the colonies turns greenish yellow. Comparable pigment is produced in Uschinsky's solution and in beef-infusion broth.



FIRURE 6.—Pure-culture isolate of a rose receptacle, from a 20-hour-old nutrient agar slant culture grown at 26° C.; stained to show flagella (Rosen Cr 1 M stain). Note polar arrangement. × 1,425.

COMPARISON OF ROSE BLAST WITH OTHER ROSE DISEASES RESEMBLING IT

As to other diseases of roses which resemble rose blast or which may be confused with it, the bacterial disease recently described very briefly by White [6] is of special interest. He noted it on greenhouse roses, particularly on the hybrid tea varieties Souvenir and Talisman. It appeared as brownish spots, often with a reddish border on leaves (blades?), on calyxes, on outer petals (in the form of "dark brown water-soaked decay"), and on young stems (small, reddish spots). He briefly notes that the bacterial colonies are gray (yellowish by transmitted light, as many grayish or whitish bacteria are likely to be), "circular with smooth regular margins and a glistening surface." So far as this description of the bacteria is concerned, it might be applied to *Phytomonas syringae*, though it is entirely too inadequate to be applied with certainty. Nothing is said about any pigment production on various culture media, and some of the symptoms as

[6] [WHITE, R. P.] A BACTERIAL DISEASE OF ROSE. N. J. Agr. Expt. Sta. Nursery Disease Notes. v. 5 (5): 1–2, 1932. [Mimeographed.]

well as the conditions under which the disease is produced suggest that it is different from rose blast.

Several other maladies which may be confused with bacterial blast of roses are of nonparasitic origin. These are (1) a browning and withering of flower buds of *Rosa rugosa* varieties and nearly all hybrid rugosas grown in the South, including F. J. Grootendorst and Sir Thomas Lipton. This the writer considers to be in the nature of species or varietal response in regions unadapted or adverse to this particular group of roses. (2) The well-known browning and blighting of many hybrid perpetuals and nearly all hybrid teas and teas which possess many petals, a malady which is seemingly associated with extremes of temperature, with perhaps the low temperatures doing most of the damage. In the South, General Jacqueminot, a great favorite of northern rose growers, is notoriously susceptible to this form of disease, while among hybrid teas Francis Scott Key, Dame Edith Helen, Jonkheer J. L. Mock, Willowmere, and Columbia not infrequently develop a "balling" and blighting of flower buds, particularly in the early part of the growing season. Various types of insect injury at times involve a browning and killing of the buds.

DISTRIBUTION OF ROSE BLAST AND OBSERVATIONS ON VARIETAL BEHAVIOR

The fact that rose blast is here reported apparently for the first time and from one locality only, suggests that it is limited in range or that it is of relatively rare occurrence. However, in view of the wide range of hosts that have been described for various isolates of *Phytomonas syringae*, if the writer's identification of the pathogen is correct it may be widely distributed, both in this country and in Europe, but limited in occurrence by weather conditions. It is to be looked for primarily in the early part of the growing season during exceptionally cool and wet weather.

As to varieties other than Magna Charta that may be susceptible to rose blast, Georg Arends, a hybrid perpetual, growing in another rose garden at Fayetteville, had flower buds that appeared to be affected with this disease. The diagnosis was uncertain, however, no culture work having been attempted. It is of interest to note that standing close to the diseased Magna Charta were a number of other rose varieties, including at least three hybrid perpetuals, Paul Neyron, Mrs. John Laing, and Frau Karl Druschki; several hybrid teas and teas, including Red-Letter Day, Madam Butterfly, Talisman, Red Radiance, Dame Edith Helen, and White Maman Cochet; and a supposed polyantha, Gruss an Aachen, which showed no symptoms of the disease. Whether this is indicative of a true resistance of these roses or whether it was merely a fortuitous occurrence cannot be stated with certainty.

SUMMARY

A disease of roses involving receptacles, calyx lobes, pedicels, and petioles is described. The disease is believed to be new to science. The malady termed "rose blast" is caused by bacteria, and the pathogen has been identified as *Phytomonas syringae*.

INHERITANCE OF RESISTANCE TO MILDEW, ERYSIPHE GRAMINIS HORDEI, IN A CROSS BETWEEN HANNA AND ATLAS BARLEY [1]

By FRED N. BRIGGS [2]

Assistant agronomist, California Agricultural Experiment Station

INTRODUCTION

Barley mildew (*Erysiphe graminis hordei*) is a common disease in California. Although usually present in fields of barley (*Hordeum Vulgare* L.), it is especially destructive to late-sown barley. Since the production of resistant varieties offers the only practical control, a study of the inheritance of resistance to mildew in hybrids between several resistant varieties and Atlas barley, which is very susceptible has been undertaken. As barley offers very good material for link, age studies, an attempt is being made to place the resistant factora in their proper linkage groups. The genetic information obtained will form the basis for a program of backcrossing by which it is expected to transfer resistance to the most important of the barley varieties grown in California. The present paper deals with data from the hybrids of Hanna × Atlas.

WORK OF OTHER INVESTIGATORS

As Hansen (*7*) [3] has recently published an extensive review of the literature dealing with the inheritance of resistance to plant diseases, only a few papers will be mentioned here.

Biffen (*1*), as early as 1907, studied the inheritance of resistance to mildew in a cross between *Hordeum spontaneum* and *H. hexasticofur catum*, the former being the most resistant and the latter the most susceptible variety in a collection of 140 varieties. The F_1 progeny was as completely attacked as the susceptible parent. In an F_2 population of 79 plants 56 were badly attacked, 16 bore a trace of the mildew, and 7 were free from it. If in addition to the 7 mildew-free plants the 16 that had a trace of the disease are considered as resistant—a conclusion which seems justified since *H. spontaneum* showed a trace of the disease under the same conditions—the result is 56 susceptible to 23 resistant. Although the number of plants was small and the results were not verified in the F_3 generation the data suggest the operation of a single recessive factor for resistance to mildew.

Dietz and Murphy (*4*) and Dietz (*3*) studied the inheritance of resistance to mildew in crosses between resistant Goldfoil C. I. 928 and four susceptible barley varieties, Chevalier C. I. 156, Trebi C. I. 936, Odessa C. I. 927, and Velvet C. I. 4252. In the four crosses

[1] Received for publication Apr. 12, 1935; issued September 1935.
[2] The writer wishes to acknowledge the assistance of L. D. Whitney and G. L. Barry in taking the mildew readings.
[3] Reference is made by number (italic) to Literature Cited, p. 249.

there were 588 susceptible and 202 resistant plants—very close to a 3:1 ratio. Taken individually, all the crosses gave ratios very close to 3:1 except Goldfoil × Velvet. Only 48 F_2 plants, however, were grown from that cross. Enough F_3 progenies were grown to verify the F_2 classifications. The factor for resistance to mildew in the Goldfoil crosses differs from that in the Hanna hybrids, because in the former susceptibility is completely dominant, as Biffen found it in *Hordeum spontaneum*, whereas, as will later be shown, susceptibility is incompletely dominant in the Hanna cross.

MATERIALS AND METHODS

In the fall of 1929 Dr. E. B. Mains, then at Purdue University, generously supplied the writer with several barley varieties known by him to be resistant to one or more physiologic forms of barley mildew. These were selected from varieties used by Mains and Dietz (*8*) in studying physiologic forms of this disease. Of the 5 forms discovered by them, Hanna C. I. 906 was completely resistant to 4 but susceptible to the fifth, giving a type 4 infection. They recognized 5 types of reaction, 0 to 4, as follows (*8, p. 231*):

> Highly resistant, denoted as 0. Macroscopically, no mycelium is evident. Chlorotic or necrotic spots may be developed by some varieties. Microscopically, a slight amount of mycelium may develop and infection may be evident macroscopically by faint flecks.
>
> Very resistant, denoted as 1. Slight to moderate development of mycelium evident macroscopically but with little or no sporulation. Chlorotic or necrotic spots developed by some varieties.
>
> Moderately resistant, denoted by 2. A moderate to abundant development of mycelium occurs, accompanied by a slight production of conidia. Chlorotic or necrotic areas are formed by some varieties.
>
> Moderately susceptible, denoted by 3. A moderate to abundant development of mycelium occurs, accompanied by moderate sporulation.
>
> Very susceptible, denoted by 4. Abundant mycelium is developed, accompanied by abundant sporulation.

The writer has followed this classification in designating the reaction of the varieties which were supplied by Mains, and which have been grown in the field at Davis, Calif., since 1930. As the 1930 crop was seeded in the fall, comparatively little mildew developed. Since that time these barleys have been seeded in late spring, and good mildew infections have resulted, although the readings have not always been so high as those obtained by Mains and Dietz in their greenhouse studies. The mildew here has corresponded very closely to their form 3, the only difference being a reading of 1 on an unnamed variety, C. I. 1021, where they obtained 0. It was interesting to find form 3, since their form 4 originally was collected in California and Idaho. According to Dietz (*3*), Dr. Lysle D. Leach collected form 4 at Davis, Calif., in 1927, 1928, and 1929.

The hybrid populations were grown in the field at Davis in the same nursery with the differential host varieties and were seeded at the same time. They were grown in rod rows 1 foot apart, the F_2 seed being spaced 6 inches part and the F_3 4 inches. Every fifth row was seeded to Atlas as a check. The disease developed entirely from natural infection. The mildew readings were made at about the time the plants were heading.

EXPERIMENTAL RESULTS

CLASSIFICATION OF F_2 AND F_3 PLANTS

From 1930 to 1934, Hanna was given a reading of 0. On a few occasions some mycelium developed, but in every case it was limited to 1 or 2 spots on an entire plant. Under the same conditions Atlas invariably was given a reading of 4.

The F_2 plants were grown in 1933. They were classified as either susceptible or resistant. Every plant on which any mildew was found was classed as susceptible. The resistant plants were tagged so that the classification could be checked in F_3. Out of a total of 386 F_2 plants, 286 were susceptible and 100 resistant. This is very close to a 3:1 ratio, with 289.5 and 96.5 the numbers expected.

Considerable variation was noted in the development of mildew on the susceptible plants, ranging from type 1 to type 4. It is believed that the lower grades of infection represent heterozygous plants, that type 4 and possibly type 3 represent homozygous susceptible plants, and that the F_2 plants could have been classified into a 1:2:1 ratio. This observation was confirmed in a study of heterozygous F_3 rows which will be discussed presently.

The F_2 data indicate, therefore, that Hanna differs from Atlas in one factor for resistance to mildew and that susceptibility is incompletely dominant.

F_3 progenies of 30 to 40 plants were grown from 384 of the 386 F_2 plants. Two plants failed to produce seed enough for an F_3 row. The F_3 results are found in table 1.

TABLE 1.—*Distribution of parent and F_2 rows of the cross named into susceptible, segregating, and resistant classes, grown in the field at Davis, Calif., 1934*

Parent or hybrid	Rows of plants		
	Susceptible	Segregating	Resistant
	Number	*Number*	*Number*
Hanna parent			15
Atlas parent	101		
Hanna × Atlas	97	193	94

The F_3 classification is very close to a 1:2:1 ratio, thus verifying the conclusion that the resistance of Hanna results from a single genetic factor. There had been a few errors in classifying the F_2 plants. In 5 cases plants marked resistant proved to be heterozygous, and 5 classified as susceptible proved resistant. This is not surprising, since even Hanna occasionally showed a trace of mildew and since in classifying the F_2 all plants showing any mycelial development were placed in the susceptible group. All homozygous susceptible plants had been correctly classified.

The segregating F_3 rows had an average of 73.9 percent susceptible plants. In the discussion of the F_2 results it was pointed out that not all the susceptible plants showed a high grade of infection, and it was suggested that heterozygous plants were intermediate in susceptibility. With this in mind a few segregating F_3 rows were classified as completely susceptible, intermediate, and resistant. The

results gave a satisfactory 1: 2 :1 ratio, which it has not seemed necessary to verify by growing a progeny test.

The F_3 data confirm the conclusion that Hanna differs from Atlas in one factor for resistance to form 3 of barley mildew, and that susceptibility is incompletely dominant.

LINKAGE STUDIES

In an extensive review of the literature on the inheritance of resistance to plant diseases Hansen (*7*) found only a few cases of linkage between disease resistance and morphological characters in plants. Griffee (*6*), studying resistance to *Helminthosporium sativum* in relation to several characters in barley, found a positive correlation between resistance versus susceptibility and the factor pairs (1) six-rowed versus non six-rowed, (2) rough versus smooth awns, and (3) white versus black glumes. Because homozygous resistant progenies are not easily recognized, Griffee (*6*) was unable to learn much about the genetics of resistance to this disease except that it was correlated with the three factor pairs mentioned above. Since these factor pairs represent 3 linkage groups, he concluded that at least 3 factors for resistance were present in the resistant variety Svanhals.

Barley offers favorable material for linkage studies because it has only seven pairs of chromosomes and the linkage between a number of factors has already been determined. Daane (*2*), in a critical survey of the literature on barley linkage, recognizes five well-established linkage groups with a number of factor pairs represented in each group.

In the present paper data are available on three pairs of factors: (1) Resistance versus susceptibility to barley mildew, (2) six-rowed versus non 6-rowed spikes, and (3) short-haired versus long-haired rachilla. Hanna is resistant to mildew, is two-rowed (*H. distichon* type), and has a long-haired rachilla. Atlas has the allelomorph to each of these characters. The two last-named factor pairs have been found by one or more investigators to give monohybrid ratios. That they segregated according to expectation may be seen in table 2.

TABLE 2.—*F_2 segregation of the factors listed in the cross of Hanna × Atlas, grown at Davis, Calif.*

Character	Observed	Expected	χ^2	P [1]
	Number	*Number*		
Susceptible to mildew	290	288	0.0139	
Resistant to mildew	94	96	.0417	
Total	384	384	.0556	>.8
Non 6-rowed	279	288	.2813	
6-rowed	105	96	.8438	
Total	384	384	1.1251	>.3
Long-haired rachilla	283	288	.0868	
Short-haired rachilla	101	96	.2604	
Total	384	384	.3472	>.5

[1] Values of P taken from Fisher (*5*).

Daane (2) place nonsix-rowed versus six-rowed in linkage group 1 and long-haired versus short-haired rachilla in group 2. That they belong to different linkage groups is confirmed by data in table 3.

TABLE 3.—*F_2 data on independent inheritance of the factor pairs listed in the cross Hanna × Atlas, grown at Davis, Calif.*

Character	Observed	Expected	χ^2	*P*
	Number	*Number*		
Non 6-rowed, long-haired rachilla	211	216	0.1157	
Non 6-rowed, short-haired rachilla	68	72	.2222	
6-rowed, long-haired rachilla	72	72	.0000	
6-rowed, short-haired rachilla	33	24	3.3750	
Total	384	384	3.7129	>0.2

The data available from Hanna × Atlas, therefore, provide a test for linkage between mildew resistance and one character each in linkage groups 1 and 2. That independent assortment occurred is shown by table 4.

TABLE 4.—*F_2 data on independent inheritance of mildew resistance vs. susceptibility with the factor pairs listed; plants grown at Davis, Calif.*

Character	Observed	Expected	χ^2	P
	Number	*Number*		
Susceptible, non 6-rowed	208	216	0.2963	
Susceptible, 6-rowed	82	72	1.3889	
Resistant, non 6-rowed	71	72	.0139	
Resistant, 6-rowed	23	24	.0417	
Total	384	384	1.7408	>.5
Susceptible, long-haired rachilla	218	216	.0185	
Susceptible, short-haired rachilla	72	72	.0000	
Resistant, long-haired rachilla	65	72	.6807	
Resistant, short-haired rachilla	29	24	1.0417	
Total	384	384	1.7409	>.5

Since no linkage was observed between mildew resistance and the characters studied in linkage groups 1 and 2, crosses are being made which involve character pairs in other linkage groups. The writer hopes to place this disease-resistant factor definitely in one of the linkage groups.

SUMMARY AND CONCLUSIONS

A study was made of the inheritance of resistance to mildew in a cross between Hanna and Atlas barley.

The results show that Hanna differs from Atlas in one factor for resistance to mildew and that suceptibility is incompletely dominant.

There was no indication of linkage between mildew resistance and one factor pair each belonging to linkage groups 1 and 2 respectively.

LITERATURE CITED

(1) BIFFEN, R. H.
1907–12. STUDIES IN THE INHERITANCE OF DISEASE-RESISTANCE. Jour. Agr. Sci. [England] 2:[109]–128, 1907; 4:[421]–429, 1912.

(2) DAANE, A.
1931. LINKAGE RELATIONS IN BARLEY. Minn. Agr. Expt. Sta. Tech. Bull. 78, 30 pp.

(3) DIETZ, S. M.
1930. THE VARIETAL RESPONSE AND INHERITANCE OF RESISTANCE IN BARLEY TO ERYSIPHE GRAMINIS HORDEI. P. F. 4. Iowa State Col. Jour. Sci. 5:25–33, illus.

(4) DEITZ, S. M., and MURPHY, H. C.
1930. INHERITANCE OF RESISTANCE TO ERYSIPHE GRAMINIS HORDEI, p. f. IV. (Abstract) Phytopathology 20: 119–120.
(5) FISHER, R. A.
1932. STATISTICAL METHODS FOR RESEARCH WORKERS. Ed. 4, rev. and enl., 307 pp., illus. Edinburgh and London.
(6) GRIFFEE, F.
1925. CORRELATED INHERITANCE OF BOTANICAL CHARACTERS IN BARLEY, AND MANNER OF REACTION TO HELMINTHOSPORIUM SATIVUM. Jour. Agr. Research 30: 915–935, illus.
(7) HANSEN, H. P.
1934. INHERITANCE OF RESISTANCE TO PLANT DISEASES CAUSED BY FUNGI, BACTERIA, AND VIRA. A COLLECTIVE REVIEW WITH A BIBLIOGRAPHY. K. Vet. og Landbohøjskole Aarsskr. 1934: 1–74.
(8) MAINS, E. B., and DIETZ, S. M.
1930. PHYSIOLOGIC FORMS OF BARLEY MILDEW, ERYSIPHE GRAMINIS HORDEI MARCHAL. Pytopathology 20: 229–239, illus.

EFFECT OF THE STAGE OF MATURITY AND METHOD OF CURING UPON THE VITAMIN B AND VITAMIN G CONTENT OF ALFALFA, CLOVER, AND TIMOTHY HAYS[1]

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INTRODUCTION

In the past hays have been produced largely with one object in mind—that of obtaining the utmost in quantity or total yield. More recently attention has been directed to the factors that affect the nutritive properties of hays, with the object of producing a product with the maximum feeding value.

Many investigators have pointed out the importance of hays as a source of vitamins for growing and producing animals. These investigations have in the main dealt with vitamins A and D. Practically no information is available regarding the vitamin G content of hays and the factors that affect it. This report presents the results of a study of the vitamin B (B_1) and G (B_2) content of alfalfa, clover, and timothy hays as they are influenced by the stage of maturity of the plant when cut and by natural climatic factors. It also includes the results of growth experiments with chicks, designed to show the value of the chick method of assay for the vitamin G complex as compared with the rat method.

REVIEW OF LITERATURE

In reviewing the literature, one finds data showing that certain practices or procedures affect the vitamin content of hays. Steenbock, Hart, and their associates (*10*)[2] found that clover hay which had been exposed to sunlight and rain was less valuable as a source of vitamin A and that it had better calcifying properties than hay which had been cured quickly and still retained a good green color. Russell (*9*) confirmed these findings, by showing that alfalfa hay dried artificially was about seven times as potent in vitamin A as hay cured in the usual way. He also noted an increase in the vitamin D content when hay was cured in the sun. Unpublished data obtained at the Ohio Station confirm the foregoing results. Bethke and Kick (*1*) observed a loss of vitamin A in alfalfa hay cured in the sun and exposed to rain and dew. Hauge and Aitkenhead (*7*) also observed a loss of vitamin A and explained that the destruction was due to enzymes. Hathaway, Davis, and Graves (*6*) found that artificially cured alfalfa was twice as potent in vitamin A as field-cured alfalfa. Douglass, Tobiska, and Vail (*5*) reported that alfalfa cut at the early bloom stage contained more vitamin A and vitamin G than hay cut at a later stage. They also reported that exposure to rain and sunshine lowered the vitamin A, vitamin B, and possibly the vitamin G

[1] Received for publication Feb. 9, 1935; issued September 1935.
[2] Reference is made by number (italic) to Literature Cited, p. 258.

content. Hunt and Krauss (*8*), in a study of pasture grasses, found that the vitamin G content decreased as the plants matured.

SOURCE OF HAYS

The alfalfa and clover hays used in this investigation were either grown on the station farm at Wooster or were obtained from the Department of Farm Crops of Ohio State University at Columbus. The timothy samples were grown on the station farm or on plots of the Timothy Breeding Station of the United States Department of Agriculture, at North Ridgeville, Ohio. Unless otherwise stated, the samples were cured indoors in subdued light.

RAT EXPERIMENTS

METHODS

In the first series of experiments for evaluating the vitamin content of the hays, rats were used as the experimental animals. These animals were born to mothers maintained on a modified Steenbock stock ration (*2*). When 24 days of age, or when weighing from 45 to 60 g, the young were placed in screen-bottom cages and were given a diet free from vitamin B and vitamin G. This diet consisted of casein (extracted if found to contain vitamin B or G), 18 percent; cornstarch, 64; hydrogenated fat (Crisco), 10; agar, 2; cod-liver oil, 2; and salt mixture (185), 4 percent.

If the rats were to be used for the determination of vitamin G each was given daily 400 mg of yeast (Northwestern), autoclaved for 4 hours at 15 pounds pressure, in addition to the basal diet. When the animals had ceased gaining in weight, which required from 14 to 21 days, they were placed in individual wire cages and fed daily the hay supplement with 400 mg of autoclaved yeast. The supplements were moistened with distilled water to prevent scattering.

If the rats were to be used for the determination of vitamin G, the basal diet was supplemented with 1 cc of an extract of rice polishings per rat daily, to furnish vitamin B. When the weights of the animals became stationary, or when they had lost slightly in weight, they were transferred to individual wire cages and the supplement (hay), moistened with 1 cc of the vitamin B preparation, was fed daily. The depletion period required on an average about 24 days.

The vitamin B extract was prepared as follows: Four kilograms of rice polishings were shaken up with 8 l of 0.1-percent hydrochloric acid and allowed to stand overnight. The clear supernatant liquid was then siphoned off. This procedure was repeated 5 or 6 times. The combined extracts were evaporated to 1,200 to 1,400 cc and then made up to 80 percent alcohol by weight. The precipitate was filtered off and washed with alcohol of the same strength. The combined alcoholic filtrates were partially concentrated under vacuum and the last traces of alcohol removed before an electric fan. The residual liquid was then diluted so that 1 cc was equivalent to 1 g of rice polishings.

The hays were ground to a fine flour, in a ball mill, for feeding purposes. The experimental feeding period in all cases was of 6 weeks' duration. The results are expressed in terms of rat units, as outlined by Bourquin and Sherman (*3*) and Chase and Sherman (*4*).

TABLE 1.—*Vitamin B and vitamin G value of hays*

VITAMIN B

Sample and no.[1]	Date cut	Rats	Level fed daily	Total gain or loss (−) in weight	Weekly gain or loss (−) in weight	Vitamin units per gram (approximate)[2]	Source and description of hay
		No.	*Milligrams*	*Grams*	*Grams*		
Alfalfa (1)	May 31, 1932	8	350	11.7	1.9	2.2	University farm; first cutting.
		8	450	22.3	3.7		
	June 10, 1932	8	400	15.3	2.5	2.0	
		8	500	18.1	3.0		
	June 21, 1932	8	500	8.1	1.5	1.6	
		8	600	18.3	3.0		
	July 28, 1932	8	400	15.8	2.6	2.0	Second cutting; injured by leaf hoppers.
		8	600	22.3	3.7		
	Sept. 10, 1932	8	450	13.3	2.2	2.0	Third cutting.
		8	500	18.2	3.0		
Alfalfa (2)	June 8, 1932	8	400	19.0	3.1	2.5	Station farm.
		8	500	26.0	4.3		
Timothy (3)	June 12, 1931	8	600	7.2	1.2	.7–0.8	U. S. Timothy Breeding Station.
	June 24, 1931	8	700	5.1	.85	[3] .6	
	July 13, 1931	8	600	−2.0	−.33	[3] .5	
	June 18, 1932	8	700	11.1	1.8	[3] 1.2	
		8	900	21.8	3.6		
Timothy (3), 15150	do	8	600	12.5	2.0	1.4	U. S. Timothy Breeding Station; late maturing.
		8	800	23.8	3.9		
Timothy (3), 12368	July 1, 1932	8	600	6.5	1.1	[3] .5	
Timothy (3)	do	8	1,000	11.5	1.9	[3] .6	Regular cutting.
Clover (1)	June 10, 1932	8	500	13.1	2.2	1.6	University farm.
		8	600	22.3	3.7		
	June 21, 1932	8	500	13.1	2.2	1.6–1.8	
		8	600	27.2	4.5		
Alfalfa from alfalfa and timothy mixture (2).	June 8, 1932	8	400	6.6	1.1	1.6	Station farm, grown as a mixed hay.
		8	600	22.2	3.7		
Timothy from alfalfa and timothy mixture (2).	do	8	600	11.4	1.9	1.2	
		8	800	24.5	4.1		
Alfalfa and timothy mixture (50:50) (2).	do	8	650	16.7	2.8	1.4–1.5	
		8	700	21.3	3.5		
400 mg autoclaved yeast (controls).		[4] 18					

VITAMIN G

Alfalfa (1)	May 31, 1932	8	100	25.1	4.2	10–12	University farm; first cutting.
		8	150	40.8	6.8		
	June 10, 1932	8	100	24.9	4.1	10.0	
	June 21, 1932	8	150	27.4	4.5	6.6	
		8	200	34.8	5.8		
	July 28, 1932	8	150	24.0	4.0	6.6	Second cutting; injured by leaf hoppers.
	Sept. 10, 1932	8	75	22.6	3.8	13.0	Third cutting.
Alfalfa (2)	June 8, 1932	8	100	15.9	2.6	6.6	Station farm; first cutting.
		8	150	16.7	2.8		Second cutting, sun-cured; no rain.
	July 21, 1932	8	100	18.5	3.0	10.0	Second cutting, sun-cured; 0.68-inch rain.
		8	200	20.7	3.4	5.0	
Timothy (3)	June 12, 1931	8	100	15.0	2.5	6.6	Heads emerging.
		8	150	24.0	4.0		
	June 24, 1931	8	150	14.2	2.3	3–4	Full bloom.
		8	300	25.4	4.2		
	July 13, 1931	8	250	13.0	2.2	2.5	Ripe, brown color.
		8	400	25.0	4.1		
	June 18, 1932	8	150	9.7	1.6	3.3	Early bloom.
		8	300	19.7	3.3		
	July 1, 1932	8	300	17.4	2.9	2–3	Late bloom.
		8	450	24.7	4.0		

[1] The same number indicates that the hays were grown in the same or in adjacent fields.
[2] A unit of vitamin B or G is the weight of the supplement which, when fed to rats under standard conditions will produce an increase in weight of 3 to 4 g per week.
[3] Estimated, animal could not consume sufficient hay to produce the required increase in weight.
[4] All rats died in 3 weeks.

TABLE 1.—*Vitamin B and vitamin G value of hays*—Continued

VITAMIN G—Continued

Sample and no.	Date cut	Rats	Level fed daily	Total gain or loss (−) in weight	Weekly gain or loss (−) in weight	Vitamin units per gram (approximate)	Source and description of hay
		No.	*Milligrams*	*Grams*	*Grams*		
Timothy (3), 15150	June 18, 1932	8	150	7.5	1.2	2–3	U. S. Timothy Breeding Station, late-maturing variety.
		8	300	16.0	2.6		
Timothy (3), 12368	July 1, 1932	8	150	9.4	1.5	3.3	Do.
		8	300	18.0	3.0		
Timothy (3), 12321	June 20, 1933	8	100	16.0	2.6	8.0	U. S. Timothy Breeding Station, late-maturing variety, sun-cured.
		8	125	19.9	3.3		
Clover (1)	June 10, 1932	8	100	24.9	4.1	10.0	University farm.
		8	150	28.7	4.8		
	June 21, 1932	8	100	27.7	4.6	10.0	
		8	200	37.8	6.3		
	June 17, 1932	8	100	17.1	2.9	8	Cured indoors.
		8	100	17.4	2.9	8	96-hour exposure, no rain.
Alfalfa from alfalfa and timothy mixture (2).	June 8, 1932	8	100	16.0	2.7	6.6	Station farm, grown as mixed hay.
		8	150	21.3	3.5		
Timothy from alfalfa and timothy mixture (2).	do	8	100	15.5	2.6	5.0	
		8	200	23.3	3.9		
Alfalfa and timothy mixture (50:50) (2).	do	8	100	16.4	2.7	6.6	
		8	200	29.0	4.8		
Controls		16		−2.0			

RESULTS

The results of the biological analyses for vitamin B, presented in table 1, show that hays are comparatively low in this factor. Alfalfa (bud stage, May 31) appeared to have a higher vitamin B content than either clover or timothy cut June 10 and 12, respectively, at approximately the same stage of maturity. The data also suggest that the vitamin B content of the plant decreases as the plant matures.

The results of the vitamin G assays (table 1) show that hays may serve as a good source of this vitamin. In general, the vitamin G content was highest in the young plant, and decreased as the plant matured. The relation of the maturity of the plant to its vitamin G content is particularly evident in the case of alfalfa and timothy; clover, however, showed no such relation. Apparently, in the case of the clover the time elapsing between the two cuttings was not long enough to bring out any differences that may have existed between the more and the less mature plants. It is of interest to note that clover and timothy may have as high a vitamin G content as alfalfa when the plants are harvested at corresponding stages of maturity. The comparative vitamin B and G content of alfalfa and timothy cut at the same time is readily seen in the case of the samples grown as mixed hay.

Leaf-hopper injury to alfalfa apparently reduces its vitamin B content. This reduction no doubt is brought about primarily by the loss of leaves. Exposure of alfalfa and clover to the weather (day and night) for 96 hours, more than one-half of which was sunshine, did not seem to affect the vitamin G content of the hay. Rain (0.68 inch), on the contrary, lowered the vitamin G content of alfalfa by 50 percent (see alfalfa (2), second cutting, table 1).

In table 2 an attempt is made to show the relationship between the protein and the vitamin B and G content of hays. It will be observed that, in general, a high protein content is correlated with a high vitamin G content. It is well known that as the plant matures the proportion of leaves to the entire plant decreases and consequently the protein and the vitamin B and G decrease. Accordingly, any procedure or process that would reduce the leaf content of the hay, such as insect injury, excessive handling, or late cutting, would lower the protein and vitamin content and also the quality of the hay.

TABLE 2.—*Protein, crude fiber, and vitamins B and G in hays*

Sample and no.[1]	Date cut	Crude fiber	Protein	Vitamin B units per gram[2]	Vitamin G units per gram[2]	Description of hay
		Percent	*Percent*			
Alfalfa (2)	May 14, 1931	17.42	22.32	2.5	13.0	60 percent leaves.
	June 10, 1931	30.02	13.83	1.8	6.6	40 percent leaves.
Alfalfa (1)	May 31, 1932	25.27	20.14	2.2	10–12	Early bud.
	June 10, 1932	32.41	17.60	2.0	10.0	Early bloom.
	June 21, 1932	34.76	15.59	1.6	6.6	Late bloom.
	July 28, 1932	32.51	12.91	2.0	6.6	Second cutting, hopper injury.
	Sept. 10, 1932	23.49	19.92	2.0	13.0	Third cutting.
Clover (1)	June 10, 1932	23.30	15.67	1.6	10.0	Early bloom.
	June 21, 1932	28.73	12.26	1.8	10.0	Late bloom.
Clover (2)	June 17, 1933	------	12.13	------	8	Sun-cured.
Timothy (3)	June 12, 1931	23.79	8.50	.7–.8	6.6	Heads emerging.
	June 24, 1931	31.05	7.25	.6	3–4	Full bloom.
	July 13, 1931	------	5.00	.5	2.5	Brown (ripe) color.
	June 18, 1932	25.65	6.65	1.2	3.3	Early bloom.
	July 1, 1932	26.97	5.21	.6	2–3	Late bloom.
Timothy from alfalfa and timothy mixture.	June 8, 1932	23.80	8.66	1.2	5.0	Grown as mixed hay.
Alfalfa from alfalfa and timothy mixture.	------do------	25.95	15.76	1.6	6.6	Do.
Mixed alfalfa and timothy (about 50:50).	------do------	24.23	10.20	1.4	6.6	Do.

[1] See footnote 1 to table 1.

[2] Approximately.

The correlation of protein and vitamin G content with greenness is only relative and varies with the amount of bleaching due to light, extent of insect injury (leaf hopper), and whether or not the plant is diseased. A similar greenness in alfalfa, clover, and timothy does not necessarily indicate the same vitamin G content. In general, however, the vitamin G values follow in the same order as the protein, leafiness, and greenness of the particular plant.

CHICK EXPERIMENTS

In order to obtain further data on the comparative value of vitamin G in different alfalfa and alfalfa-leaf meals, day-old chicks were used as the experimental animals. These experiments gave a direct comparison of the value of the chick and rat methods of testing for vitamin G.

White Leghorn chicks from the same parent stock were used. They were confined in pens equipped with hardware cloth floors, and fed a ration known to be deficient in vitamin G. Each lot contained 25 chicks. The basal ration was composed of yellow corn, 58 percent; ground wheat, 20; wheat bran, 5; casein (Argentine), 12; steamed bone meal, 3; salt, 1; and cod-liver oil, 1 percent. The meals were incorporated in this ration in the amounts indicated in table 3. The casein and corn were so adjusted that the protein content was approximately the same in all lots. The meals used represented composite

samples of three or more lots, with the exception of the Ohio leaf meals, which involved only one sample. The Ohio leaf-meal fed to lots 10 and 11 (table 3) was prepared from sun-cured hay that had received 0.68 inch of rainfall while in the swath. Liver meal is considered to be a very rich source of vitamin G and for that reason one lot of chicks was fed 3 percent of this as a positive control.

TABLE 3.—*Effect of alfalfa, alfalfa-leaf, and liver meals on growth and leg paralysis of chicks*

Lot no.	Supplement	Protein in meal	Meal consumed per chick in 8 weeks	Rat units of vitamin G per gram of meal	Total rat units of vitamin G consumed per chick in 8 weeks	Average weight per chick in 8 weeks	Leg paralysis	
							Total [1]	Recovered [2]
		Percent	*Gram*			*Gram*	*Percent*	*Percent*
1	None					168.8±4.8	67.0	0.0
2	5 percent alfalfa meal [3]	16.0	38.7	8.0	309.6	278.8±14.0	68.0	8.0
3	10 percent alfalfa meal [3]	16.0	118.5	8.0	948.0	466.8±13.3	29.0	17.0
4	5 percent alfalfa-leaf meal [3]	20.25	49.9	13.0	648.7	384.6±15.2	48.0	12.0
5	10 percent alfalfa-leaf meal.[3]	20.25	130.6	13.0	1,697.8	517.3±11.8	.0	
6	5 percent alfalfa meal [4]	14.25	35.6	8.0	284.8	251.9±11.3	87.0	35.0
7	10 percent alfalfa meal [4]	14.25	119.7	8.0	957.6	454.9±15.0	28.0	16.0
8	5 percent alfalfa-leaf meal [4]	20.56	55.2	13.0	717.6	438.3±14.4	48.0	48.0
9	10 percent alfalfa-leaf meal [4]	20.56	132.4	13.0	1,721.2	528.3±9.9	.0	
10	5 percent alfalfa-leaf meal [3] (0.68 inches rain).	20.31	32.8	5.0	164.0	240.6±11.9	83.0	33.0
11	10 percent alfalfa-leaf meal [3] (0.68 inches rain).	20.31	107.1	5.0	535.5	397.9±13.6	36.0	20.0
12	3 percent dried pork liver					602.9±12.3	.0	

[1] The figures in this column indicate the percentage of chicks that developed leg paralysis during the experiment.
[2] The figures in this column indicate the percentage of chicks that recovered before the end of the experiment.
[3] Meal from hays grown in Ohio.
[4] Meal from hays grown in Colorado.

It is evident from the data presented in table 3 that the growth of the chicks and the incidence of leg paralysis are directly correlated with the vitamin G content of the meals as determined with rats. Leaf meals having a higher protein and vitamin G content than straight meals caused greater growth and less incidence of leg paralysis than the regular meals of lower protein and vitamin G content. The poor growth and comparatively large percentage of leg paralysis in the case of lots 10 and 11, which received 5 and 10 percent respectively of the leaf meal subjected to rain, suggest that a large part of the vitamin G was lost, and corroborate the results obtained with rats. The data also show that alfalfa meals and leaf meals of approximately the same vitamin G potency produce comparable responses in chicks, whether the meals are produced in Ohio or in Colorado.

A second experiment was conducted partly to check the results of the first trial and partly to compare meals prepared from clover and timothy with those of alfalfa. The same basal ration and procedure used in the first test were employed. The meals were incorporated in the basal ration in amounts shown in table 4. The casein and corn were so adjusted that the total protein content of the rations in all lots

was comparable. The clover and timothy meals were prepared from single samples of hay, while the alfalfa meals represented composite samples of three or more lots. Twenty chicks were started in each lot.

The results presented in table 4 show the ineffectiveness of 10 percent of the straight hay meals in preventing leg paralysis-substantiating the results obtained in the first experiment. Although the results show some variation between different meals, the data in general reveal a close correlation between the response in growth and incidence of leg paralysis and the vitamin G content of the meals as determined on rats. Clover meal gave results comparable to alfalfa meals of the same vitamin G potency but a higher protein content. The results obtained with the timothy meals also compare favorably with those of alfalfa when the vitamin G content of the meals is considered. As in the previous experiment, there was no difference in the results with the Ohio and Colorado meals when compared on the same vitamin G basis.

TABLE 4.—*Effect of alfalfa, clover, timothy, and liver meals on growth and leg paralysis of chicks*

Lot no.	Supplement	Protein in meal	Meal consumed per chick in 8 weeks	Rat units of vitamin G per gram of meal	Total rat units of vitamin G consumed per chick in 8 weeks	Average weight per chick in 8 weeks	Leg paralysis	
							Total [1]	Recovered [2]
		Percent	*Gram*			*Gram*	*Percent*	*Percent*
1	None					176.0±5.3	95.0	15.0
2	5 percent clover [3]	11.54	38.0	8.0	304.0	243.0±12.3	85.0	35.0
3	10 percent clover [3]	11.54	125.7	8.0	1,005.6	453.5±20.2	15.5	0.0
4	5 percent timothy [4]	7.63	38.9	5.0	194.5	261.8±9.0	65.0	5.0
5	10 percent timothy [4]	7.63	100.2	5.0	501.0	345.5±11.8	70.0	45.0
6	10 percent timothy [5]	5.62	92.3	3.0	276.9	314.0±10.6	70.0	25.0
7	5 percent alfalfa meal [6]	12.25	48.2	8.0	385.6	306.0±11.1	80.0	35.0
8	10 percent alfalfa meal [6]	12.25	110.8	8.0	886.4	389.8±13.2	40.0	5.0
9	10 percent alfalfa meal [7]	14.25	101.1	6.6	667.2	368.7±11.9	45.0	35.0
10	5 percent alfalfa meal [7]	15.56	49.6	8.0	396.8	325.9±11.1	55.0	25.0
11	10 percent alfalfa meal [7]	15.56	125.7	8.0	1,005.6	430.0±20.2	20.0	10.0
12	3 percent dried pork liver					612.6±9.1	5.0	5.0

[1] The figures in this column indicate the percentage of chicks that developed leg paralysis during the experiment.
[2] The figures in this column indicate the percentage of chicks that recovered before the end of the experiment.
[3] See table 1. Clover cut June 17; 96 hours exposure.
[4] Timothy cut June 9 (heads emerging). Cured in sun.
[5] Cut in late-bloom stage.
[6] Meal from hays grown in Colorado.
[7] Meal from hays grown in Ohio.

The data in tables 3 and 4 show a close correlation between the total consumption of vitamin G and the weight of the chick and incidence of leg paralysis. The figures in the column showing the total consumption do not include the vitamin G content of the basal ration.

The data obtained by the method used in this investigation appear to show that 10 percent of an alfalfa-leaf meal containing 13 rat units of vitamin G per gram is necessary to induce good growth and prevent the occurrence of leg paralysis.

SUMMARY AND CONCLUSIONS

Alfalfa, clover, and timothy hays cut at different times and cured under different conditions were tested for vitamin B and G with rats as the experimental animals. The results show that these hays contain significantly more vitamin G than vitamin B. The vitamin B and vitamin G content of the hays decreased as the plant matured and, in general, were correlated with the leafiness, greenness, and protein content of the plant.

The exposure of alfalfa to the weather (day and night) for 96 hours—over half of which was sunshine—without rain, did not affect the vitamin G content. Rain (0.68 inch), on the contrary, removed as much as 50 percent of this vitamin.

Timothy and clover cut early may have as high a vitamin G content as alfalfa cut later, and with a much greener color.

The method of testing for vitamin G by growth and incidence of leg paralysis in chicks compared favorably with the rat-assay method.

Ten percent of an alfalfa leaf meal containing 13 rat units of vitamin G per gram was required to induce good growth in chicks and prevent the occurrence of leg paralysis.

LITERATURE CITED

(1) Bethke, R. M., and Kick, C. H.
1929. vitamin-a content of alfalfa hay. Ohio Agr. Expt. Sta. Bull. 431 (Ann. Rept. 47): 117–118.

(2) ——— Steenbock, H., and Nelson, M. T.
1923. fat-soluble vitamins. xv. calcium and phosphorus relations to growth and composition of blood and bone with varying vitamin intake. Jour. Biol. Chem. 58: 71–103.

(3) Bourquin, A., and Sherman, H. C.
1931. quantitative determination of vitamin g (B_2). Jour. Amer. Chem. Soc. 53: 3501–3505, illus.

(4) Chase, E. F., and Sherman, H. C.
1931. a quantitative study of the determination of the anti-neuritic vitamin b. Jour. Amer. Chem. Soc. 53: 3506–3510, illus.

(5) Douglass, E., Tobiska, J. W., and Vail, C. E.
1933. studies on changes in vitamin content of alfalfa hay. Colo. Agr. Expt. Sta. Tech. Bull. 4, 68 pp., illus.

(6) Hathaway, I. L., Davis, H. P., and Graves, R. R.
1932. the vitamin a and the vitamin e contents of field-cured and artificially cured alfalfa hay. Nebr. Agr. Expt. Sta. Research Bull. 62, 15 pp.

(7) Hauge, S. M., and Aitkenhead, W.
1931. the effect of artificial drying upon the vitamin a content of alfalfa. Jour. Biol. Chem. 93: 657–665, illus.

(8) Hunt, C. H., and Krauss, W. E.
1931. the influence of the ration of the cow upon the vitamin b and vitamin g content of milk. Jour. Biol. Chem. 92: 631–638, illus.

(9) Russell, W. C.
1929. the effect of the curing process upon the vitamin a and d content of alfalfa. Jour. Biol. Chem. 85: 289–297.

(10) Steenbock, H., Hart, E. B., Elvehjem, C. A., Kletzien, S. W. F., and Rising, B. M.
1925. dietary factors influencing calcium assimilation. vi. the antirachitic properties of hays as related to climatic conditions, with some observations on the effect of irradiation with ultra-violet light. Jour. Biol. Chem. 66: 425–440. illus.

THE INFLUENCE OF CERTAIN DIETARY CONSTITUENTS ON THE RESPONSE OF RATS TO GOSSYPOL INGESTION[1]

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INTRODUCTION

Chemical methods for the quantitative determination of gossypol in cottonseed products are based upon the formation of an insoluble compound of gossypol with aniline. In the determination of the gossypol content of raw cottonseeds, the gossypol and oil are extracted from the seeds with diethyl ether. After evaporation of the ether, the residue is taken up with petroleum ether and the gossypol is precipitated with aniline. A positive correlation between the toxicity of raw cottonseeds and their gossypol content has been established by the use of this method (*10*).[2]

It has not been possible to establish a similar correlation between the gossypol content and the toxicity of heated cottonseeds or cottonseed meal, principally for two reasons; (1) Only a small portion of the gossypol can be extracted from heated seeds or the meal with the usual fat solvents, and (2) the insoluble portion (so-called D-gossypol or bound gossypol) which may be extracted with hot aniline is of questionable toxicity (*4*). It has been necessary, therefore, in investigating the gossypol content of these heated cottonseed products to resort to biological methods of assay and from the results so obtained to approximate the amount of physiologically active gossypol which they contain.

In a preliminary study of various methods of biological assay the response of rats to gossypol was found to vary widely depending on the method of administration of the substance (per os and intraperitoneally) and apparently on the nutritive condition of the animals. The most promising method of assay and one of general application was based upon a comparison of the growth rate of rats on diets containing the product under investigation with that of rats on diets of known gossypol content. Later studies revealed that rats were unusually susceptible to gossypol injury while on diets low in vitamin A (*6*), and in view of the instability of gossypol and its detoxication by soluble iron salts (*5*) there was reason to believe that the effect of the substance on growth might vary with the nature of the diet in which it was incorporated.

The primary purpose of the present study was to determine what effect alterations in the basal diet might have upon the growth response of rats to gossypol ingestion. Such information was of particular importance in formulating a standard procedure of biological assay. A second purpose, possibly of greater practical importance, was to determine whether certain dietary supplements might act similarly to iron salts in effecting a partial if not complete detoxication of gossypol.

[1] Received for publication Apr. 1, 1935; issued September 1935.
[2] Reference is made by number (italic) to Literature Cited, p. 266.

METHODS AND EXPERIMENTAL DATA

EXPERIMENTS WITH DIETS OF VARYING PROTEIN CONTENT

The possibility that the deleterious effects of gossypol might be lessened or increased in diets of high protein content was considered first. Accordingly, experimental diets in which the protein was progressively increased from approximately 13 percent to 51 percent were prepared by replacing starch with appropriate amounts of casein. Gossypol was incorporated in the diets by including 15 percent of cottonseeds containing 0.35 percent gossypol. From previous studies it was calculated that this amount of gossypol would not be lethal but would allow the majority of the rats to make slow growth. The percentage composition of the diet of lowest protein content was: Casein 7.4, starch 60.6, Crisco 5.0, salts 3.5, yeast 5.0, cod-liver oil 3.5, and raw cottonseeds 15. The casein was increased to 22, 35, and 51 percent in the diets of higher-protein content.

Control diets of corresponding protein content were prepared in the same way, cottonseeds from which the gossypol had been extracted with ether being used. The oil so removed from the seeds was replaced by an equivalent amount of refined cottonseed oil. The only important difference between the control and the experimental diets so prepared was in their gossypol content. Because of the unstable nature of gossypol, it was deemed necessary to prepare new diets from freshly ground seeds each week.

Young rats weighing between 50 and 60 g which had made uniform growth over a period of a week or 10 days were divided according to litter origin and sex between the gossypol and the control diets. Precautions were taken in selecting the rats to obtain uniform animals and to reduce to a minimum variation in their growth which might be attributed to inheritance or previous nutritional state. For the most part they represented the second, third, and fourth litters of a selected group of healthy females which had been reared and bred for the investigation. They were housed in small individual cages with raised screen bottoms set over circles of paper fitted in the bottom of granite pans. Food was supplied in weighed amounts in feed cups especially constructed to reduce waste. Daily records were kept of the food intake.

As the experiment progressed it was found necessary, because of idiosyncrasies shown by a few animals, to discontinue those rats which at the end of 30 days failed to establish a gain of 30 g. Such animals usually succumbed to the effects of gossypol or lost weight during the next few weeks. Otherwise, the animals were contained on the experimental and control diets for a period of from 60 to 90 days. The results are presented in table 1. The figures for food consumption and gains in weight are average values obtained with an equal number of males and females.

From the results given in table 1, it is apparent that an increase in the protein content of the diet from 13 to 35 percent resulted in an improved rate of growth of the animals on both the gossypol and control diets. A further increase of protein to 51 percent proved less effective for growth of the controls, although on this high level of protein the growth rate of the animals receiving gossypol remained approximately the same as that of those on 35 percent of protein. It is also significant that with a diet containing 35 percent of protein a

large percentage of the animals on the gossypol diets were able to establish the required gain of 30 g during the first 30 days.

It will be observed in table 1 that on each level of protein the animals receiving gossypol consumed less food than did the corresponding controls, although their food intake increased as the amount of protein in the diet was increased to 35 percent. A decrease in food intake is a characteristic effect of gossypol and is attributed not so much to the taste of the compound as to its physiological effect.

To eliminate the effect upon growth resulting from differences in the food intake of the experimental and control rats, the same diets were employed in paired-feeding experiments in which the daily food intake of the animals on the control diet was limited to that of the animals receiving gossypol. The differences in the gains in weight made by the experimental and control animals in each pair in these experiments are, therefore, to be attributed to the effect of gossypol and are unrelated to the plane of nutrition. The results are presented in table 2.

TABLE 1.—*Effect of gossypol in the diet on growth of rats, as influenced by the protein content of the diet when the animals were permitted to eat ad libitum*

Approximate percentage of protein in diet	Gossypol content of diet	Rats started on experiment	Rats continued on experiment	Average food consumed in 60 days	Average gain in weight in 60 days	Difference in gains in weight[1]
	Percent	*Number*	*Number*	*Grams*	*Grams*	*Grams*
13	0.05	14	10	413	69	64
	(2)	7	7	632	133	
26	.05	13	10	485	96	56
	(2)	8	7	663	152	
35	.05	11	9	497	110	60
	(2)	5	5	712	170	
51	.05	13	10	433	108	42
	(2)	7	7	570	150	

[1] In favor of the rats receiving the gossypol-free diet.
[2] Control, no gossypol.

TABLE 2.—*Effect of gossypol in the diet on growth of rats, as influenced by the protein content of the diet when the animals were on paired-feeding tests*

Pair no.[1]	Protein content of diet	Gossypol in diet	Duration of experiment	Total food consumed	Total gain in weight	Difference in gain between pair mates[2]	Pair no.[1]	Protein content of diet	Gossypol in diet	Duration of experiment	Total food consumed	Total gain in weight	Difference in gain between pair mates[2]
	Pct.		*Days*	*Grams*	*Grams*								
1 M	13	Yes	60	418	77	10	9 M	35	Yes	90	924	202	7
		No	60	422	87				No	90	926	209	
2 F	13	Yes	60	535	78	26	10 F	35	Yes	90	796	172	15
		No	60	536	104				No	90	798	187	
3 M	13	Yes	60	432	62	32	11 M	35	Yes	90	826	205	5
		No	60	433	94				No	90	827	210	
4 F	13	Yes	60	376	57	12	12 M	35	Yes	90	873	183	17
		No	60	377	69				No	90	872	200	
5 M	26	Yes	90	898	155	30	13 F	51	Yes	60	392	93	14
		No	90	898	185				No	60	393	107	
6 F	26	Yes	90	670	95	13	14 M	51	Yes	60	536	157	16
		No	90	665	108				No	60	535	173	
7 M	26	Yes	90	723	154	39	15 F	51	Yes	60	386	72	27
		No	90	714	193				No	60	387	99	
8 F	26	Yes	90	623	113	24	16 M	51	Yes	60	503	142	6
		No	90	624	137				No	60	502	148	

[1] Letter following number indicates sex of animals.
[2] In favor of the rat receiving the gossypol-free diet.

A comparison of the difference in gains in weight between pair mates on the diets of high and low protein content as shown in table 2 affords further evidence of the beneficial effect of a relatively high percentage of protein in diets containing gossypol. Upon a diet containing 35 percent of protein, animals receiving gossypol were able to approximate the gains in weight made by their litter mates on the control diet. At the end of 60 days the average gain in weight made by the male rats on the gossypol diet containing 35 percent of protein was 169 g. As in the preceding experiments, the diets containing 51 percent of protein were less favorable to growth than those containing 35 percent.

These results suggest the possible detoxication of gossypol by protein. Detoxication of gossypol by iron salts is believed to be due to the formation of an iron-gossypol complex which escapes digestion, and it is possible that protein or its degradation products detoxicates gossypol in a similar way. Support for this idea is found in the results of digestion studies carried out in vitro by Jones and Waterman (*7*), who found that the peptic and tryptic digestion of protein was inhibited by gossypol. Schwartze and Alsberg (*11*) observed that the addition of gossypol to the diet of cats resulted in an increased fecal nitrogen. It has also been suggested that a combination of gossypol with protein accounts for the insoluble condition of gossypol in cottonseed meal (*2*, *12*). None of the evidence so far obtained, however, has been sufficiently conclusive to settle the point. It may be that in the present experiment the increased growth obtained with diets containing 35 percent of protein was due largely to the increased nutritive value of the diets. Nevertheless, considerable importance is attached to these results since they suggest a relationship between protein intake and gossypol injury and explain in part the observations of Clark (*3*), who found that increasing the casein content of cottonseed-meal diets resulted in an increased rate of growth.[3]

EXPERIMENTS WITH DIETS OF VARYING FAT AND LACTOSE CONTENT

In view of the favorable results obtained with diets of high protein content, it appeared possible that alterations in the amount of other dietary constituents might likewise prove beneficial.

To determine the effect of altering the fat content of the diet, experimental diets 23, 11, and 22, containing 15 percent of cottonseeds and having a fat content of approximately 7, 12, and 22 percent, respectively, and control diets 11a and 22a, containing 12 and 22 percent of fat, were employed. These diets were fed to groups of rats selected in the manner described in the first experiment. The composition of the diets is given in table 3.

The results of the experiments, which are given in table 4, indicate that a slight improvement in growth is made possible by the inclusion of a large amount of fat in the diet of animals receiving gossypol. The differences in gain in weight between the gossypol and control rats on the high-fat diet and between similar groups of rats on the medium-fat diet were 51 and 56 g, respectively. The differences between the average gains made by the rats on the low- and high-fat diets containing gossypol were not considered sufficiently significant,

[3] Since these experiments were completed, Robison (*9*) has reported the results of studies begun in 1928 to determine the value of cottonseed meal as a protein supplement for growing pigs. As a result of his studies he has set forth the tentative hypothesis that the harmful effect of feeding cottonseed meal to pigs is averted when tankage or a similar protein concentrate is fed, because the gossypol of the meal combines with the protein of the tankage or similar feed to form an insoluble and therefore harmless material.

however, to justify further experimentation with diets of variable fat content. As might be expected, the amount of food required per gram of gain in body weight markedly decreased as the fat content of the diet was increased.

It has been demonstrated that lactose is responsible for a change in intestinal absorption, due possibly to the maintenance of an acid reaction in the intestinal tract (*1*). The insolubility of gossypol in weak acid suggested the possibility that the addition of lactose to a diet containing gossypol might prove beneficial. Gossypol and control diets containing 20 and 35 percent of lactose were, therefore, employed. Unsatisfactory results were obtained with the higher percentage of lactose due to the development of diarrhea. The results obtained with 20 percent of lactose in gossypol diet 46 and in control diet 46a are given in table 4. From these results it is evident that the addition of lactose not only failed to improve the growth rate of animals receiving gossypol, but produced a decrease in the growth rate of the control animals.

TABLE 3.—*Composition of diets used* [1]

Components and constituents of diets	Composition [2] of diet no.—						
	23	11	22	40 [3]	46 [3]	26	28
Components:	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Raw cottonseeds	15.0	15.0	15.0	10.0	10.0	15.0	15.0
Starch	50.7	45.7	35.7	49.0	29.0	44.1	42.7
Casein	22.3	22.3	22.3	24.0	24.0	22.3	22.3
Yeast	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Cod-liver oil	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Salt mixture [4]	3.5	3.5	3.5	3.5	3.5	----	3.5
Crisco	----	5.0	15.0	5.0	5.0	5.0	5.0
Lactose	----	----	----	----	20.0	----	----
Acid salt mixture [5]	----	----	----	----	----	3.0	----
Calcium chloride	----	----	----	----	----	1.1	----
Calcium carbonate	----	----	----	----	----	1.0	2.0
Sodium bicarbonate	----	----	----	----	----	----	1.0
Constituents:							
Gossypol	.053	.053	.053	.060	.060	.053	.053
Calcium	.430	.430	.430	.428	.428	1.225	1.230

[1] For each experimental diet made up with raw cottonseeds containing gossypol, a control diet of like composition was made up with gossypol-free seeds. These latter diets are designated in the text and table 4 by the letter a following the number of the diet.

[2] The potential acidity of diets 23, 11, 22, 40, and 46 was equivalent to 170 to 190 cc of N/10 alkali. The potential acidity of diet 26 was equivalent to 447 cc of N/10 alkali. The potential basicity of diet 28 was equivalent to 349 cc of N/10 acid.

[3] The gossypol content of the raw seeds used in diets 40 and 46 was 0.60 percent.

[4] McCollum's salt mixture no. 185 (*8*).

[5] The same as McCollum's salt mixture no. 185 except that the calcium lactate (1.30 g) called for in this mixture was replaced by 0.650 g of calcium chloride.

TABLE 4.—*Effect of gossypol on growth of rats as influenced by the fat and lactose content and the reaction of the diet when the animals were permitted to eat ad libitum*

Diet no.	Character of diet	Gossypol in diet	Rats started on experiment	Rats continued on experiment	Average food consumed in 60 days	Average gain in weight in 60 days	Difference in gain in weight [1]
			Number	*Number*	*Grams*	*Grams*	
23	Low fat	Yes	6	5	426	97	----
11	Medium fat	Yes	13	10	485	96	56
11a [2]	do	No	8	7	663	152	
22	High fat	Yes	12	9	393	104	51
22a [2]	do	No	9	9	446	155	
46	20 percent lactose	Yes	8	7	417	81	44
46a [2]	do	No	6	6	728	125	
40	No lactose	Yes	21	17	468	85	75
40a [2]	do	No	6	6	629	160	
26	Acidic	Yes	11	7	415	101	24
26a [2]	do	No	11	10	500	125	
28	Basic	Yes	10	9	503	128	33
28a [2]	do	No	6	6	636	161	

[1] In favor of the rat receiving the gossypol-free diet.

[2] Control diet; see footnote 1, table 3.

EXPERIMENTS WITH ACIDIC AND BASIC DIETS

To determine the possible influence of potentially acidic and basic diets on the growth response of rats to gossypol ingestion, calcium chloride and calcium carbonate were added in amounts calculated to yield diets of equal calcium content but of a definitely acid reaction in the one case and alkaline in the other. The amount of calcium chloride used in the first diets tested was too large to permit any of the animals to make consistent gains. The diets rapidly developed a rancid odor and it was found necessary to replace a part of the chloride with an amount of calcium carbonate which would reduce the acidity and yet maintain the calcium content of the diet at the desired level. Rancidity was prevented in these diets by withholding the acid salt mixture from the bulk of the diet and intimately mixing it with each day's supply. The composition of the acidic and basic diets which were finally used and which were found to give the most consistent results is presented in table 3 as diets 26 and 28.

An inspection of the results in table 4 reveals that although the diets made acid by the addition of calcium chloride allowed a majority of the rats on gossypol to make a slight improvement in growth rate they actually proved detrimental to the growth of the control animals. Were it not for the fact that of the 11 animals started on the acidic gossypol diet, 4 were unable to grow during the first 30 days of the experiment, potentially acid diets high in calcium might be considered favorable to the detoxication of gossypol. For example, the control rats on diet 26a made an average gain of only 125 g in 60 days, which was 27 g less than that established by other controls on a similar diet containing less potential acid (diet 11a). However, the gossypol rats on diet 26 made an average gain of 101 g, which was only 24 g less than that made by their controls and 5 g more than that made by rats on a gossypol diet of the same composition exclusive of the acid salt (diet 11).

On the other hand, the use of an alkaline diet prepared by the inclusion of 2 percent of calcium carbonate and 1 percent of sodium bicarbonate (diets 28 and 28a) and having the same calcium content as the acidic diets effected a marked improvement in the growth rate of the rats receiving gossypol and a slight improvement in the growth of their controls. The food intake of gossypol and control animals was increased as a result of this alteration in the diet and it is to be noted that these alkaline diets promoted greater gains and allowed a larger percentage of the animals to survive the experiment than had been possible with any of the previous diets employed. In this dietary series the growth rate of the gossypol animals more closely approached that of the controls than was true in any of the other series with but one exception, that being the acid series, in which the controls made unusually small gains. Of the 10 rats started on the alkaline gossypol diet, 9 finished the experiment with an average gain of 128 g, which was only 33 g less than the average gain made by their controls and 32 g more than that made by rats on a similar diet (diet 11) unsupplemented with the basic salts.

As previously pointed out, precautions had been taken to preserve the integrity of the gossypol molecule in preparing the various diets. Seeds of known gossypol content were used instead of a purified preparation of the isolated substance and fresh diets were frequently pre-

pared. It does not seem likely that destruction of gossypol by its contact with alkali or other reactive constituents in the diet occurred previous to ingestion. Rather, the results lead to the opinion that calcium salts are favorable to the detoxication of gossypol in the animal organism and are particularly effective in the presence of excess base. The possible relationship between calcium metabolism and susceptibility to gossypol injury has been alluded to in an earlier paper (*6*). Further evidence in support of this opinion has been obtained in experiments carried out in vitro in which gossypol, when dissolved in oil and suspended in a buffered alkaline solution, was precipitated upon the addition of a soluble calcium salt. The stability of this calcium complex has not been determined.

SUMMARY AND CONCLUSIONS

The necessity of using biological methods of assay for the determination of physiologically active gossypol in certain cottonseed products prompted a study of dietary factors which influence the response of rats to gossypol ingestion. Accordingly, experimental diets containing a known percentage of gossypol were varied in their content of protein, fat, carbohydrate (lactose), and calcium and in their potential acidity and alkalinity. For each of these alterations made in the gossypol diets, a similar alteration was made in a control gossypol-free diet. The criterion used to judge the effectiveness of a dietary alteration in modifying the deleterious effect of gossypol was that the effect so produced be more apparent in the performance of the rats on gossypol diets than in their controls.

The results of ad libitum and paired feeding experiments with diets of variable protein content (13 to 51 percent) indicated that a high protein intake was favorable to the detoxication of gossypol. The effect of high fat diets was questionable. Lactose when fed at a 20-percent level proved to be detrimental to the growth of normal rats; its effect on the gossypol rats was slight. Acidic diets high in calcium (1.2 percent) were likewise detrimental to the growth of normal rats and only slightly influenced the growth of rats which received gossypol and survived the experiment. Alkaline diets high in calcium (1.2 percent) proved to be superior to any of the others in allowing the rats on gossypol to approximate a normal growth rate.

On the basis of these results the conclusion is reached that calcium salts in the presence of excess base are especially favorable to the detoxication of gossypol in the animal organism. Indirect evidence of a chemical reaction between calcium and gossypol is offered in support of this conclusion.

The recommendation is made that in the biological assay of gossypol, a basal diet be employed which is potentially slightly acid and contains only that amount of calcium and protein which is compatible with maintenance and moderate growth. It is tentatively suggested that in feeding cottonseed products to secure rapid growth the ration should contain a reasonable excess of the base-forming elements, particularly calcium, and liberal amounts of protein.

LITERATURE CITED

(1) Bergeim, O.
1926. intestinal chemistry. v. carbohydrates and calcium and phosphorus absorption. Jour. Biol. Chem. 70: 35–45.
(2) Clark, E. P.
1928. studies on gossypol. ii. concerning the nature of carruth's d gossypol. Jour. Biol. Chem. 76: 229–235.
(3) ———
1929. studies on gossypol: a progress report. Oil and Fat Indus. 6(7): 15–19, illus.
(4) Gallup, W. D.
1928. relation of d-gossypol to the toxicity of some cottonseed products. Indus. and Engin. Chem. 20: 59–63.
(5) ———
1928. the value of iron salts in counteracting the toxic effects of gossypol. Jour. Biol. Chem. 77: 437–449.
(6) ———
1931. studies on the toxicity of gossypol. i. the response of rats to gossypol administration during avitaminosis. Jour. Biol. Chem. 93: 381–405.
(7) Jones, D. B., and Waterman, H. C.
1923. studies on the digestibility of proteins in vitro. iv. on the digestibility of the cottonseed globulin and the effect of gossypol upon the peptic-tryptic digestion of proteins. Jour. Biol. Chem. 56: 501–511, illus.
(8) McCollum, E. V., and Simmonds, N.
1918. a study of the dietary essential, water-soluble b, in relation to its solubility and stability towards reagents. Jour. Biol. Chem. 33: 55–89, illus.
(9) Robison, W. L.
1934. cottonseed meal for pigs. Ohio Agr. Expt. Sta. Bull. 534, 44 pp., illus.
(10) Schwartze, E. W., and Alsberg, C. L.
1924. relation between toxicity of cottonseed and its gossypol content. Jour. Agr. Research 28: 173–189, illus.
(11) ——— and Alsberg, C. L.
1924. pharmacology of gossypol. Jour. Agr. Research 28: 191–198, illus.
(12) Withers, W. A., and Carruth, F. E.
1918. gossypol, the toxic substance in cottonseed. Jour. Agr. Research 12: 83–102, illus.

STUDIES ON THE NICOTINE CONTENT OF CIGARETTE SMOKE[1]

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INTRODUCTION

Nicotine has been praised and condemned ever since it was first isolated from tobacco by Posselt and Reimann as cited by Pictet (19a, *p. 159*)[2] in 1828. Both praise and condemnation have had little factual basis, however, for the physiological action of nicotine in tobacco smoke is still a controversial subject. Before investigators can establish the effect of inhaling nicotine in smoke, they must know how much of the alkaloid is present. Many conflicting statements are found on this subject.

A review of the literature shows that several factors may cause a fluctuation in the nicotine content of tobacco smoke, among which are the moisture content of the tobacco, the rate of smoking, the quantity left unburned, and the nicotine content of the tobacco itself.

In order to study these factors a satisfactory method for the determination of nicotine in smoke must be employed. The existing methods were therefore reviewed.

METHODS FOR DETERMINING NICOTINE IN TOBACCO AND TOBACCO SMOKE

A method for the determination of nicotine in tobacco smoke must have two particular qualifications. As in the case of tobacco itself, the method must not include the estimation of ammonia, pyridine and its derivatives, or other basic substances as nicotine. It is particularly important that ammonia be separated quantitatively from the nicotine, as shown by the data of Haley, Jensen, and Olson (*8*)[2] on cigar smoke. In fact, the ammonia content of smoke equals or exceeds the nicotine content in some cases.[3] Schaarschmidt (*20*) has shown that pyridine is present in much smaller amounts.

A second qualification which the method must have is the ability to measure accurately small amounts of nicotine.

Picric acid reacts with nicotine to give the yellow amorphous dipicrate which gradually changes to the crystalline form. Pfyl and Schmitt (*19*) have used this reaction as the basis of a method for the determination of nicotine. This method with modifications was used by several German investigators (*2, 6, 12, 18, 26*), in the analysis of both tobacco and tobacco smoke. Pfyl and Schmitt state that the picric acid method is excellent for the determination of nicotine in tobacco smoke because ammonia, pyridine, and other bases do not interfere. But Koperina (*12*) states that nitrogen compounds do

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[2] Reference is made by number (italic) to Literature Cited, p. 274.

[3] JENSEN, C. O. THE CHEMISTRY OF TOBACCO SMOKE. I. THE STUDY OF THE NICOTINE AND AMMONIA CONTENT OF CIGAR SMOKE. Thesis, M. S., Pa. State Col. 1930.

interfere in this determination. In a comparison of the Pfyl and Schmitt method (*19*) with Baggesgaard-Rasmussen's silicotungstic acid method (*1*), Heiduschka and Muth (*9*) showed that the two methods gave practically identical results.

At the present time Chapin's silicotungstic acid method (*7*) or modifications of it are widely used for nicotine determinations (*3*, *23*, *4*, *11*, *16*, *22*, *23*).

Any method in which silicotungstic acid is used as the precipitating agent solves the question of ammonia separation because ammonium silicotungstate is soluble in water (*7*). Pyridine is more likely to interfere in the precipitation of nicotine (*7*, *14*, *17*). However, Mach and Sindlinger (*14*) state that nicotine can be separated from pyridine in cold 0.5 percent hydrochloric acid solution, if the solution is made so dilute that no pyridine is precipitated. In this case only a very small amount of nicotine escapes precipitation. Baumberger (*3*) has stated that the nicotine content of smoke can be determined by Chapin's method without pyridine interference. Wenusch (*25*) used this method on cigar smoke and found that the results obtained were too high. On diluting the solutions to 10 times their original volume before precipitation, the results were the same as those obtained on the polarimeter. It was possible for Wenusch to determine nicotine by the polariscope for he used the smoke from 25 cigars for one determination.

Because of the possible erroneous results that might be obtained if too much pyridine is present, a series of determinations were made to find the concentration at which pyridine does not interfere with the preceipitation of nicotine in dilute solutions.

Samples containing nicotine and added amounts of pyridine were treated as outlined by Chapin (*7*). One hundred cubic centimeters of a solution of nicotine and pyridine was placed in a Kjeldahl flask, made alkaline with sodium hydroxide, and steam-distilled. The solutions obtained were made up to 500 cc, and four 100-cc aliquots were taken. The first aliquot was not diluted but the other three were diluted 1–1, 1–2, and 1–3 with distilled water, acidified with HCl, before precipitation with 5 cc of 12 percent silicotungstic acid. The precipitates were filtered through weighed Gooch crucibles, dried for 3 hours at 125° C., and weighed. The results are given in table 1.

TABLE 1.—*Determination of nicotine as influenced by various concentrations of pyridine*

Solution analyzed	Volume of solution precipitated	Dilution	Concentration of added pyridine	Concentration of nicotine	Weight of precipitate	Nicotine calculated on basis of original sample
	cc		*Percent*	*Percent*	*Grams*	*Percent*
0.060 g nicotine in 100 cc, no pyridine	100	None.	0	0.012	0.1164	0.059
	100	None.	0	.012	.1168	.059
	200	1–1	0	.006	.1141	.058
	300	1–2	0	.004	.1105	.056
	500	1–4	0	.002	.1070	.054
0.060 g nicotine and 0.20 pyridine in 100 cc	100	None.	.04	.002	.1649	.083
	200	1–1	.02	.006	.1188	.060
	300	1–2	.013	.004	.1104	.056
	500	1–4	.008	.002	.1075	.054
0.060 g nicotine and 0.060 g pyridine in 100 cc	100	None.	.12	.012	.5666	.286
	200	1–1	.06	.006	.3014	.153
	300	1–2	.04	.004	.1649	.083
	500	1–4	.024	.002	.1052	.053

These results show that in solutions containing less than 0.02 percent of pyridine, there is little interference in the estimation of nicotine. However, it is also evident that as the solutions are diluted a small amount of nicotine escapes precipitation. In order, therefore, to be certain that pyridine does not interfere with the determination of nicotine, the solutions must be diluted by trial until there is no sharp drop in the weight of the precipitate.

Solutions obtained from the absorption of cigarette smoke were so treated, and there was no significant difference between the weight of the precipitates from the diluted and the undiluted samples. This agrees with the results of Baumberger (*3*) and Schaarschmidt and his coworkers (*20*). Furthermore, after 18 hours the precipitates were crystalline and settled out rapidly after stirring, which is not the case when more than 0.02 percent of pyridine is present.

Since ammonia did not interfere with the results of Chapin's silicotungstic acid method (*7*) and pyridine did not interfere under the conditions of the writers' tests with rather dilute smoke solutions, this method was used for the present work.

APPARATUS AND PROCEDURE

The machine shown in figure 1 was devised to smoke cigars, cigarettes or pipes with a constant length and strength of puff at the same intervals.

The cigarette *a* is held in a small calcium chloride tube *b* and the smoke is absorbed in the gas-absorption tubes *c*, *d*, *e*, and *f*. The pump *p* creates a partial vacuum in the flask *j*, and this puffs the cigarette whenever the slowly rotating valve *h* is open.

The reducing gear *g* is connected by a short piece of garden hose to the valve *h*. The reducing gear is turned by a synchronous motor *i*. In order to prevent an increase of the vacuum in *j* as measured by the water manometer *m*, air is allowed to bubble through *l* into the bottle *k* which contains oil. The amount of vacuum can be regulated by the height of the oil in *k* and also partly by the valve *n*. The calcium chloride tower *o* is used to prevent water vapor from entering the pump *p*.

Cigarettes were stored for at least 2 weeks in containers of known humidity so that the moisture content could be controlled. Five cigarettes were weighed to the nearest hundredth of a gram and smoked with a "puff" 1.6 seconds long with an interval of 6.1 seconds between puffs. The difference between atmospheric pressure and the pressure in the vacuum bottle was 15.25 inches of water, unless otherwise stated. This allowed 20 cc of air, as measured by a gas meter, to pass through the cigarette with every puff. The smoke was collected in a train of four gas-absorption tubes each containing 12.5 cc of chloroform and 12.5 cc of 0.1 N sulphuric acid.

After the smoke from the five cigarettes had been collected the chloroform-acid solution was placed in a separatory funnel together with the chloroform and water washings from the cigarette holder and the absorption tubes. The liquid was agitated and the lower chloroform layer was removed and discarded, since nicotine sulphate is insoluble in chloroform. The acid solution containing the nicotine sulphate was placed in a Kjeldahl flask and steam-distilled after the addition of 35 cc of sodium hydroxide and a few pieces of porous plate.

The volume of liquid at this point was about 300 to 400 cc. In order to keep the volume of the solution from increasing the flask was heated gently. The distillate was collected in water acidified with 15 cc of dilute HCl (1–4) until 500 cc had distilled over. After the distillation was completed the nicotine was precipitated with 5 cc of 12 percent silicotungstic acid, stirred well, and allowed to stand overnight. It was then filtered through a weighed Gooch crucible, heated in an oven at 125°C. for 3 hours, and weighed. The weight of the precipitate multiplied by the factor 0.1012 gave the weight of nicotine. This was divided by the weight of tobacco smoked to get the weight of nicotine secured from 1 g of tobacco. The data given in this paper are averages of two or more results.

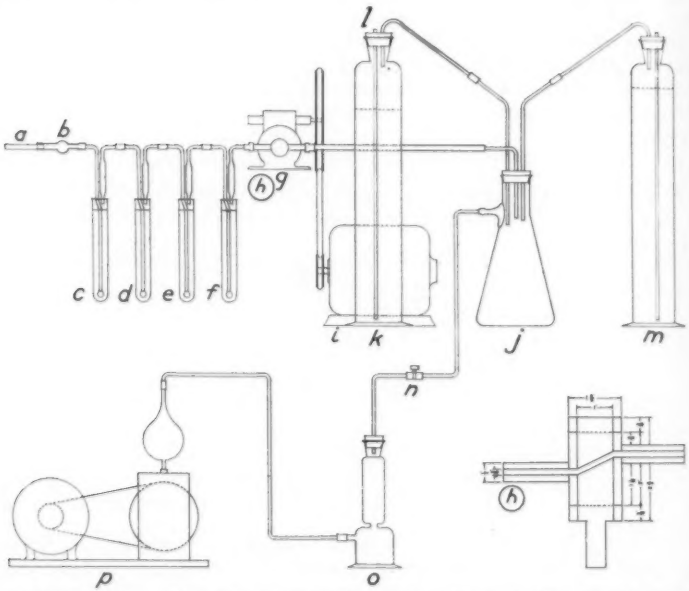

FIGURE 1.—Apparatus used to smoke cigars, cigarettes, or pipes with constant length and strength of puff at same time intervals: *a*, Cigarette; *b*, small calcium chloride tube used as cigarette holder; *c-f*, glass-stoppered gas-absorption tubes, 1 by 8 inches; *g*, reducing gear, 48 to 1, with 8-inch pulley; *h*, brass valve; *i*, synchronous motor, one-sixteenth horsepower, 1,200 revolutions per minute; *j*, 2-liter suction flask; *k*, tall (24-inch) bottle containing motor oil, S. A. E. 30, to a height of 17 inches; *l*, 3.5-mm glass air-inlet tube; *m*, tall (24-inch) bottle used as a water manometer; *n*, Hoke needle valve from oxygen tank; *o* calcium chloride tower; *p*, high-vacuum pump.

The experimental conditions did not of course duplicate actual cigarette smoking conditions.

NICOTINE CONTENT OF CIGARETTE SMOKE AS AFFECTED BY THE MOISTURE CONTENT OF THE TOBACCO

In 1923 Heinz (*10*) stated that smoke from a moist cigar contained from 50 to 75 percent more nicotine than smoke from a dry cigar. But Schöller's results (*21*) in 1928 showed practically no difference between the nicotine content of the smoke of dry and moist cigars.

Winterstein and Aronson (*29*) published results in 1929 which differed from the findings of both of these workers. They state that from a dry cigarette 30 percent more nicotine goes into the mouth of the smoker than from a moist one. The water content of the dry tobacco was 4.8 percent and that of the moist tobacco was 16.5 percent. Waser and Stähli (*24*) and Molinari (*15*) also found more nicotine in the smoke from dry cigarettes than from moist ones. Results which differed from any yet reported were given by Kovalenko (*13*) in 1931, who stated that an increase in the moisture content of cigarettes from 9 to 11 percent increases the nicotine content of the smoke but a further increase in the moisture content decreases the nicotine in the smoke.

EXPERIMENTS

Cigarettes were stored for a period of at least 2 weeks in desiccators having known humidities. The desired relative humidities were obtained from the data of Wilson (*27*) on humidity control by means of sulphuric-acid solution.

The moisture content of the cigarettes was determined by drying in a vacuum over concentrated sulphuric acid for 2 weeks. The procedure described in the previous section was used to determine the nicotine content of the smoke from these cigarettes. The results are shown in table 2.

TABLE 2.—*Nicotine content of cigarette smoke as affected by the moisture content of the tobacco*

Moisture content of cigarette (percent)	Nicotine found in smoke per gram of dry tobacco	Nicotine found in the smoke as compared to nicotine in the original tobacco	Moisture content of cigarette (percent)	Nicotine found in smoke per gram of dry tobacco	Nicotine found in the smoke as compared to nicotine in the original tobacco
	Milligrams	*Percent*		*Milligrams*	*Percent*
0.0	9.2	42.4	11.10	5.7	26.3
3.99	7.8	35.9	14.34	5.1	23.5
6.43	7.3	33.6	24.44	4.6	21.2
9.22	6.5	30.0			

These results clearly show that an increase in the moisture content of cigarettes decreases the nicotine content of the smoke. Contrary to the findings of Kovalenko (*13*), the nicotine found in the smoke does not increase with the moisture content from 9 to 11 percent.

NICOTINE CONTENT OF CIGARETTE SMOKE AS AFFECTED BY THE STRENGTH OF PUFF

Bogen (*5*), Wenusch (*26*), and Kovalenko (*13*) all state that the nicotine content of cigarette smoke increases with the rate of smoking.

EXPERIMENTS

Cigarettes stored at four different humidities were smoked under conditions identical with those reported in the previous section, except that the vacuum used was equal to 14 inches of water instead of 15.25 inches. This allowed 16 cc of air to pass through the cigarette at

each puff instead of 20 cc as in the previous series. The results are given in table 3.

TABLE 3.—*Nicotine content of cigarette smoke as affected by the strength of puff, cigarettes of different moisture content being used* [1]

Moisture content of cigarette (percent)	Nicotine found in the smoke per gram of dry tobacco		Nicotine found in the smoke as compared to nicotine in the original tobacco	
	16 cc of air puff	20 cc of air puff	16 cc of air puff	20 cc of air puff
	Milligrams	*Milligrams*	*Percent*	*Percent*
0.0	8.1	9.2	37.3	42.4
3.99	7.0	7.8	32.3	35.9
6.43	6.6	7.3	30.4	33.6
31.5	2.3	.....	10.6	.....

[1] In these trials 16 cc of air passed through the cigarette at each puff instead of 20 cc as in series reported in table 2.

The results show that an increase in the volume of air going through the cigarette at each puff increases the amount of nicotine in the smoke. This agrees with the findings of others (*5*, *13*, *26*).

NICOTINE CONTENT OF CIGARETTE SMOKE AS AFFECTED BY THE LENGTH OF BUTT

Heiduschka and Muth (*9*) determined the amount of nicotine in the smoke when four-fifths of a cigarette was moked. Using a 4-second puff at 6-second intervals, they found an average of 0.19 percent of the weight of the cigarettes as nicotine in the smoke. The original nicotine content of the cigarettes used was 1.19 percent.

EXPERIMENTS

Cigarettes of 7-cm length with a nicotine content of 2.17 percent and a moisture content of 11.1 percent were smoked until butts of 1-, 2-, or 3-cm lengths remained. The cigarettes were given a light coating of paraffin near the end and inserted in a warm glass tube which was only slightly larger than the cigarettes. Only the part to be burned remained outside the tube. The paraffin solidified on cooling, forming an air-tight joint. The cigarettes were smoked until the burning zone reached the glass tube. The results are given in table 4.

TABLE 4.—*Nicotine content of cigarette smoke as affected by the length of butt*

Length of butt (length of unsmoked cigarettes, 7 cm) (centimeters)	Fraction of cigarette smoked (F)	Amount of nicotine in smoke per cigarette whose dry weight equals 1 g (A)	Nicotine found in the smoke as compared to nicotine in the original tobacco	Nicotine condensed in butt $\left(\frac{5.7F-A}{5.7F}\times 100\right)$
		Milligrams	*Percent*	*Percent*
3	4/7	1.3	6.0	60.1
2	5/7	2.5	11.5	38.6
1	6/7	3.8	17.5	22.3
0	7/7	5.7	26.3	0.0

The percentage of nicotine condensed in the butt was calculated from the difference between the amount of nicotine actually found and the nicotine which theoretically should have been found in the smoke $\left(\frac{5.7\ F-A}{5.7\ F}\times 100\right)$.

The condensation of nicotine in the unburned tobacco is a very important factor governing the amount of the alkaloid that the smoker will receive. Under the conditions of this experiment the condensation amounts to 60 percent of the nicotine which ordinarily appears in the smoke, when the length of unburned cigarette is 3 cm or three-sevenths of the original product. With a length of butt of 1 cm, only 22 percent of the nicotine which ordinarily appears in the smoke was held in the unburned portion. The actual amount of nicotine found in the smoke of 1 cigarette burned to a length of 1 cm will be approximately equal to the nicotine found in the smoke from 3 cigarettes whose butts are 3 cm in length. One cigarette smoked to a 2-cm butt will have as much nicotine in its smoke as 2 cigarettes smoked to a length of 3 cm. Although the above exact relationships will hold true only under the conditions of this experiment, it is apparent that the length of butt is one of the most important factors governing the nicotine content of cigarette smoke.

THE NICOTINE CONTENT OF THE "SIDE STREAM"

Bogen (*5*) states that the side stream ordinarily constitutes the greater part of the smoke as shown by the carbon-dioxide content. Winterstein and Aronson (*28*) measured the nicotine and reported that 43 to 62 percent of the total nicotine goes into the side stream.

EXPERIMENTS

Cigarettes were placed in the machine and smoked as usual, but instead of allowing the side stream to escape, it was trapped and passed through four gas-absorption tubes. In order to trap the side stream the burning cigarette was placed in a bulb with two small openings at opposite sides. Through one of these openings the cigarette was placed. This opening had a diameter 1 cm greater than that of the cigarette holder, which allowed air to enter the bulb at such a rate that none of the smoke was lost. The air and smoke were pulled through the other opening into the gas-absorption tubes by means of a continuously running water pump.

The results are given in table 5.

TABLE 5.—*Nicotine content of the side stream smoke as modified by the moisture content of the cigarette*

Moisture content of cigarette (percent)	Main stream (amount of nicotine as compared to the nicotine in tobacco)	Side stream (amount of nicotine as compared to the nicotine in tobacco	Amount of nicotine in tobacco not found in smoke
	Percent	*Per cent*	*Percent*
11.13	26.3	31.8	41.9
0.0	42.4	28.6	29.0

Contrary to the results of the study of the relation between the moisture content of the tobacco and the amount of nicotine in the main stream, the side stream shows a decrease of nicotine when the moisture content decreases. This may be partly explained by the fact that dry cigarettes burn more quickly than moist ones. Only 8 puffs are required to burn 1 cm of the dry cigarettes, while 15 puffs are required for those with a moisture content of 11.13 percent. In the more rapidly burning dry cigarettes there is evidently greater distillation and less destruction of the nicotine. This accounts for the increased amounts of the alkaloid in the main stream. But the rate at which the tobacco burns when air is not passing through the dry cigarette may not be increased to the extent that it is when air is drawn through it. Thus the shortened time of burning will cause a smaller amount of nicotine to be found in the side stream. A study of the carbon-dioxide content of the two streams of smoke and the temperature of the burning zone might help to explain the foregoing facts.

SUMMARY

A study of methods of determining nicotine, applicable to cigarette-smoke solutions, has shown (1) that pyridine does not interfere in the precipitation of nicotine by silicotungstic acid in concentrations below 0.02 percent, and (2) that the concentration of pyridine in cigarette-smoke solutions is not high enough to interfere with the precipitation of nicotine by silicotungstic acid.

A machine is described which will smoke cigarettes, cigars, or pipes with puffs of constant volume and unvarying length at constant intervals.

The nicotine content of cigarette smoke varies inversely as the moisture content of the cigarettes.

The amount of nicotine in the smoke is directly proportional to the strength of the puff.

There is a marked condensation of nicotine in the short unburned portion of a cigarette.

Under the conditions of these experiments cigarettes with a moisture content of 11.13 percent contained more nicotine in the side stream than in the main stream; cigarettes with a moisture content of 0 contained less nicotine in the side stream than in the main stream.

LITERATURE CITED

(1) Baggesgaard-Rasmussen, H.
1915. die bestimmung von nicotin in tabak und tabakprapäraten. Abstract in Chem. Ztg. 39: 25.

(2) Barta, L., and Toole, E.
1931. mikrotitrimetrische bestimmung des nicotins im tabakrauch. Ztschr. Angew. Chem. 44: 682–683, illus.

(3) Baumberger, J. P.
1923. the nicotine content of tobacco smoke. Jour. Pharmacol. and Expt. Ther. 21: 35–46, illus.

(4) Benvegnin, L.
1931. du dosage de la nicotine par la méthode au silicotungstate. Mitt. Lebensmtl. Untersuch. u. Hyg. [Switz.] 22: 217–220. [Abstract in Chem. Abs. 26: 1390. 1932.]

(5) Bogen, E.
1929. the composition of cigarets and cigaret smoke. Jour. Amer. Med. Assoc. 93: 1110–1114.

(6) Bolm, F.
1930. Über rauchen, nicotingrenzzahlen und die nicotinbestimmung nach pfyl und schmitt. Ztschr. Untersuch. Lebensmtl. 59: 602–606.
(7) Chapin, R. M.
1911. The determination of nicotin in nicotin solutions and tobacco extracts. U. S. Dept. Agr., Bur. Anim. Indus. Bull. 133, 22 pp.
(8) Haley, D. E., Jensen, C. O., and Olson, O.
1931. A study of the ammonia content of cigar smoke. Plant Physiol. 6: 183–187, illus.
(9) Heiduschka, A., and Muth, F.
1928. Über nikotin im tabak ii. Pharm. Zentralhalle 69: [305]–307.
(10) Heinz, R.
1923. Ueber die giftigkeit des tabakrauches, insbesondere des zigarettenrauches. Deut. Med. Wchnschr. 49: 318–319.
(11) Kasansky, B.
1931. Massanalytische schnellbestimmung von nicotin mittels kiesel-wolframsäure. Ztschr. Analyt. Chem. 83: 107–114.
(12) Koperina, A. W.
1930. Untersuchung der stickstoffhaltigen verbindungen des tabakrauches. Biochem. Ztschr. 219: 258–276, illus.
(13) Kovalenko, E.
1931. The nicotine content of tobacco smoke. State Inst. Tobacco Invest. (U. S. S. R.) Bull. 81: 77–85. [Abstract in Chem. Abs. 26: 6066. 1932.]
(14) Mach, F., and Sindlinger, F.
1924. Versuche zu bestimmung des pyridins mit kieselwolframsäure, insbesondere bei gegenwart von nicotin. Ztschr. Angew. Chem. 37: 89–92.
(15) Molinari, E.
1932. Über den einfluss der luftfeuchtigkeit auf den nikotingehalt des tabakrauches. Fachl. Mitt. Österr. Tabakregie 1932(2): 4–7.
(16) Nagy, V. L.
1932. Bestimmung kleiner nicotinmengen im tabakrauch. Biochem. Ztschr. 249: [404]–408.
(17) North, E. O., and Beal, G. D.
1924. The preparation, properties, and uses of silicoduodecitungstic acid. Jour. Amer. Pharm. Assoc. 13: 889–898.
(18) Petri, W.
1930. Zur beurteilung des nicotingehalts der tabake. Ztschr. Untersuch. Lebensmtl. 60: 123–136.
(19) Pfyl, B., and Schmitt, O.
1927. Zur bestimmung von nicotin in tabak und tabakrauch. Ztschr. Untersuch. Lebensmtl. 54: 60–78, illus.
(19a) Pictet, A.
1904. The vegetable alkaloids. With particular reference to their chemical constitution. From 2d Fr. ed., rendered into Engl., rev. and enl. . . . by H. C. Biddle. 505 pp. New York and London.
(20) Schaarschmidt, A., Hofmeier, H., and Nowak, P.
1932. Untersuchungen über die verwendbarkeit von adsorptionsmitteln zum entgiften von tabakrauch. Chem. Ztg. 56: 911–913, illus.
(21) Schöller, R.
1928. Nikotingehalt des rauches feuchter und trockener zigarren. Fachl. Mitt. Österr. Tabakregie 1927–28 (2): 2–4.
(22) Wagner, O.
1932. Eine standardmethode zur bestimmung des nicotins. Chem. Ztg. 56: 462–463, illus.
(23) Wakeham, G.
1930. Rapid nephelometric method for the approximate determination of nicotine in tobacco. Chemist Analyst 19(1): 8–10.
(24) Waser, E., and Stähli, M.
1932. Untersuchungen am tabakrauch. I. Ztschr. Untersuch. Lebensmtl. 64: 470–485.

(25) WENUSCH, A.
1927. NIKOTINBESTIMMUNGEN IN DEN RAUCHGASEN NORMALER UND ENTNIKOTINISIERTER ZIGARREN. Fachl. Mitt. Österr. Tabakregie 1927–28(1): 7–8.
(26) ———
1931. BEITRAG ZUR BESTIMMUNG DES NIKOTINS IN RAUCHGASEN. Fachl. Mitt. Österr. Tabakregie 1931(1): 1–8.
(27) WILSON, R. E.
1921. HUMIDITY CONTROL BY MEANS OF SULFURIC ACID SOLUTIONS, WITH CRITICAL COMPILATION OF VAPOR PRESSURE DATA. Jour. Indus. and Engin. Chem. 13: 326–331, illus.
(28) WINTERSTEIN, A., and ARONSON, E.
1928. BEITRÄGE ZUR KENNTNIS DES TABAKGENUSSES. II. MITTEILUNG. ÜBER DEN VERBLEIB DES NICOTINS BEIM TABAKRAUCHEN. Ztschr. Hyg. u. Infektionskrank. 108: [530]–553, illus.
(29) ——— and ARONSON E.
1929. UEBER DAS ZIGARETTENRAUCHEN. Schweiz. Med. Wchnschr. 59: 550–552.

THE EFFECT OF AUXINS UPON PHYTOPHTHORA CACTORUM[1]

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INTRODUCTION

Ever since Pasteur announced that yeasts were unable to grow in a solution consisting of mineral salts and sugar, investigators have devoted much time not only to the effect of such substances on yeasts and yeastlike fungi, but also to the effect of growth-promoting factors extracted from yeast and tested upon plants and animals. An extensive survey of literature on this subject is given by Miller (*6*),[3] Tanner (*12*), Sherman and Smith (*11*), and Buchanan and Fulmer (*2*). With a few exceptions, filamentous fungi have received but little attention. What few fungi have been tested belong, largely, to the usual genera, such as *Penicillium*, *Aspergillus*, and *Rhizopus*. For instance, Kögl and his coworkers (*5*), who have isolated three different auxins from human urine and have determined their chemical structure, report the finding of growth-promoting substances in several species of *Rhizopus* as well as in *Escherichia coli*. Nielsen (*7*) found an auxin in *Rhizopus suinus* and named it rhizopin. According to him this substance accelerates growth of *Aspergillus niger* nine times and greatly increases conidia production. Nielsen and Hartelius (*8*) made further studies of rhizopin and reported that it contains two distinct auxins, A and B. Auxin A is soluble in ether, is destroyed by oxidation, and promotes growth in oat coleoptile just as Kögl's auxins do. Auxin B, on the other hand, is insoluble in ether, is not destroyed by oxidation, and promotes growth in *A. niger* and not in oat coleoptile. Von Euler and Philipson (*4*) report that oat coleoptiles contain a growth factor for *R. tritici*, *R. nigricans*, *Aspergillus wentii*, and a species of *Penicillium*. Almoschlechner (*1*) classifies the auxin found in *Boletus edulis* as "Wuchsstoffe B" which, like auxin B of Nielsen and Philipson, is insoluble in ether. Schopfer (*10*) found that *Phycomyces blakesleanus* would not grow in a mineral solution and a pure grade of sugar, whereas commercial maltose induced good growth. He tested the effect of vitamins B^1 and B^2 of Windaus and Kuhn and found that these substances induced growth in the fungus as well as in wheat seedlings.

It is quite probable that a great many fungi are capable of manufacturing their own auxins, while others depend upon other organisms for their growth-promoting substances. For instance, *Mucor genevense*, *Zygorhynchus heterogamus*, *Pythium butleri*, and *Pythium debaryanum* make a good growth in a mineral solution and sugar, whereas *Phycomyces blakesleanus*, *Pythium polymastum*, *Phytophthora cactorum*, and *Coprinus radians* cannot grow unless some complex organic substance is added to the nutrient solution.

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[2] The writer is indebted to Dr. Virgil G. Lilly for suggestions concerning the chemical phase of the work.

[3] Reference is made by number (italic) to Literature Cited, p. 286.

EXPERIMENTS

This paper is concerned only with *Phytophthora cactorum* (Leb. and Cohn) Schroeter. The fungus makes no growth whatever when transferred to a solution consisting of 5 g of pure dextrose, 1 g of potassium nitrate, 0.5 g of dihydrogen potassium phosphate, and 0.25 g of magnesium sulphate in 1,000 cc of water. When, however, the primary root of an aseptically germinating corn grain is cut when it is about an inch long and is transferred to this solution, the fungus grows readily (fig. 1) and reproduces both asexually and sexually.

Some may object to the composition of the nutrient solution described above on the ground that it does not contain such essential elements as zinc, manganese, boron, iron, copper, iodine, thallium, etc., which, while needed in very minute quantities, may, nevertheless, be controlling factors in the growth of fungi. In order to overcome this objection, some canned green peas were dried and ashed at a comparatively low temperature; the ash was treated with hydro-

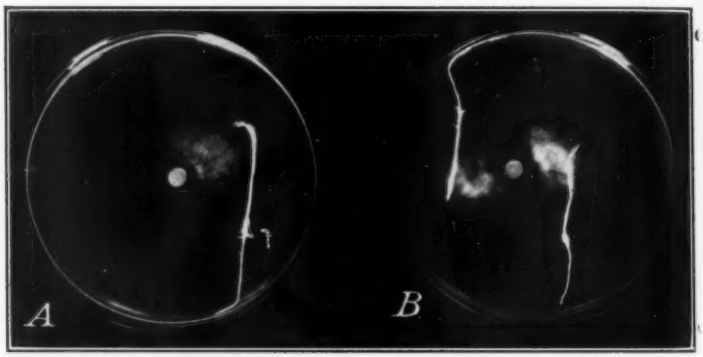

FIGURE 1.—Effect of corn root auxins upon *Phytophthora cactorum*: *A*, Growth one-sided because there is only one source of auxins; *B*, A more symmetrical growth because of the presence of two sources of auxins. Note that growth is toward the base of the root where the auxins are given off.

chloric acid, evaporated to dryness on a steam bath, and added to the nutrient solution. It could induce no growth whatever despite the fact that canned peas induce richer and faster growth in *Phytophthora cactorum* than any other substance with which the writer has had experience.

The corn root does not need to remain in the solution for very long; even so short an exposure as 1 minute is sufficient to endow the solution with a certain degree of growth-promoting quality. The longer the root is allowed to remain in the solution, the better the growth will be. But this is not at all proportionate. For instance, the same piece of root was successively transferred through a number of flasks, each containing 15 cc of the nutrient solution. The root was kept in the first flask for only 1 minute, 5 minutes in the second, 10 in the third, 15 in the fourth, 30 in the fifth, 1 hour in the sixth, 2 hours in the seventh, 4 in the eighth, 8 in the ninth, 16 in the tenth, and 24 hours in the eleventh flask. The amount of growth made in the last flask was only three times that made in the first flask. The same was true when a different root was used for each flask.

Went (*14*) and others have shown that auxins have a definite polarity in the coleoptile of sprouting oat grains, most of the auxins being concentrated at the tip and from there moving toward the base. Popov (*9*) obtained similar results with corn sprouts. Thimann (*13*) states that in the roots of *Avena* the concentration of auxins decreases with distance from tip. Cholodny (*3*) finds that in the case of corn roots transport of growth-promoting substances occurs only in the basal direction. The writer also found that the movement of growth-promoting substances was toward the base, and comparatively smaller amounts were present near the growing tip. This was demonstrated by the following methods. Fifteen cubic centimeters of nutrient medium prepared according to the foregoing formula and solidified by means of 0.75 percent of a pure grade of agar-agar was poured in a series of Petri dishes and sterilized. In the first series of dishes the roots of corn were so placed as to bring the cut base in direct contact with the agar, the remaining parts of the roots being supported above the surface of the agar by means of short pieces of glass tubing. In the second series the position of the root was reversed, and the cut base of the root was sealed with paraffin in order to check any possible diffusion of protoplasmic substances to the surface and from there into the agar. In the third series the position of the root was the same as in the second but the root tip was cut off and the cut end was allowed to rest on the agar. In the fourth series, the position of the root was like that in the first except that the root tip was cut off and sealed with paraffin. All these dishes were then inoculated. The results are shown in figure 2. It can be seen that when the cut base of the root was allowed to come in direct contact with the agar, it induced very good growth; the presence or the absence of the root tip did not seem to have any appreciable effect upon the amount of these substances diffused into the agar. Least growth was made when the sound root tip rested upon the agar; the removal of the root tip caused a somewhat better growth.

Despite the paraffin coating, a certain amount of cell fluid worked its way out from under the paraffin and accumulated on the root in the form of an amber-colored drop. Whenever this drop fell on the agar before it could be absorbed by means of a filter paper, a rich growth ensued. The following technique was used to overcome this: Corn grains were germinated aseptically; when the primary root was about 4 inches long and the shoot had formed, the young plant was removed to a small flask containing the nutrient solution. Only the tip of the root was allowed to come in contact with the solution. In some cases the root tip was cut off; in others it was left intact. The sound roots gave off only a trace of growth-promoting substances and the fungus produced but a slight growth; where the root tip was cut off, a somewhat better growth resulted, but in no case could it compare with the growth induced by a short piece of root aseptically removed from a germinating corn grain and transferred to the nutrient solution. In another series of experiments the corn grains, as soon as they showed signs of germination, were transferred to Petri dishes containing the nutrient medium solidified with agar. The agar was then inoculated; the primary roots were allowed to attain a length of 2 inches. The dishes were then divided into two groups. In the first group the tip of the primary root was

cut off, while in the second group in was left intact. In the case of the former some growth resulted, while in the case of the latter no growth whatever could be seen.

The relative concentration of growth-promoting substances in the roots was tested by another method. When the primary root was about an inch in length, it was cut away from the grain and then cut into two equal parts; the basal portion was transferred to one flask containing the nutrient solution, and the apex to another flask; the

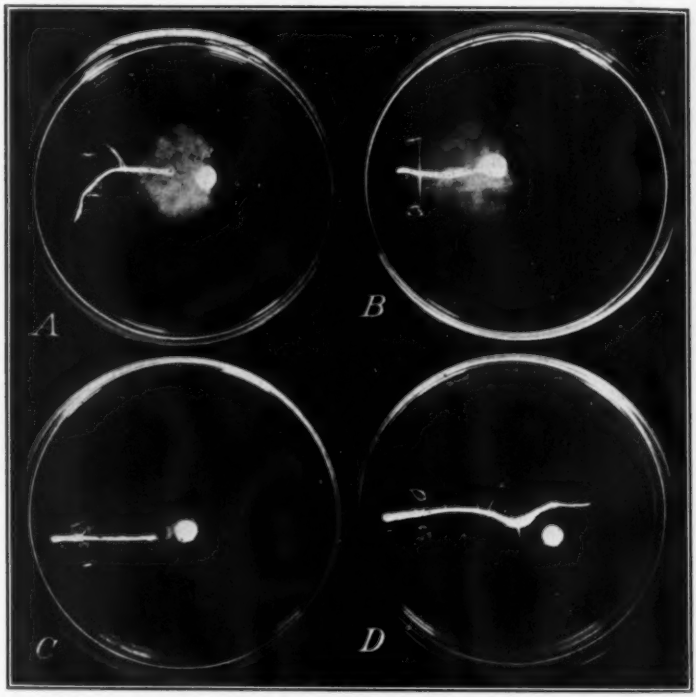

FIGURE 2.—Polarity of auxins: *A*, The base of the root resting on agar, all other parts being elevated by means of a glass tubing, the root tip intact; *B*, the base of the root resting on agar, tip cut off; *C*, the apical portion of the root, with the tip removed, resting on the agar, while the base is elevated; *D*, the sound apical portion of the root resting on the agar.

flasks were then inoculated. In all cases the basal portion induced a much better growth; even when the root tip of the apical portion was removed and thus two wounded regions were exposed to the solution, no appreciable difference could be seen.

The growth-promoting substances alone, as given off by the roots, cannot support growth. When a piece of root and an inoculum were placed in a dish containing 2 percent agar-agar, no growth resulted. The addition of potassium nitrate, dihydrogen potassium phosphate, and magnesium sulphate, alone or in all possible combinations, induced no growth despite the presence of the root. But the root and dextrose together induced a fairly good growth. However, the

maximum growth resulted only in the presence of the essential salts, glucose, and the root (fig. 3). If the root was left out, no growth resulted.

In all Petri-dish preparations the inoculum was always placed about an inch away from the root. It was observed that the growth of the fungus was always towards the root, so that eventually a fan-shaped colony resulted. No matter how long the fungus was allowed

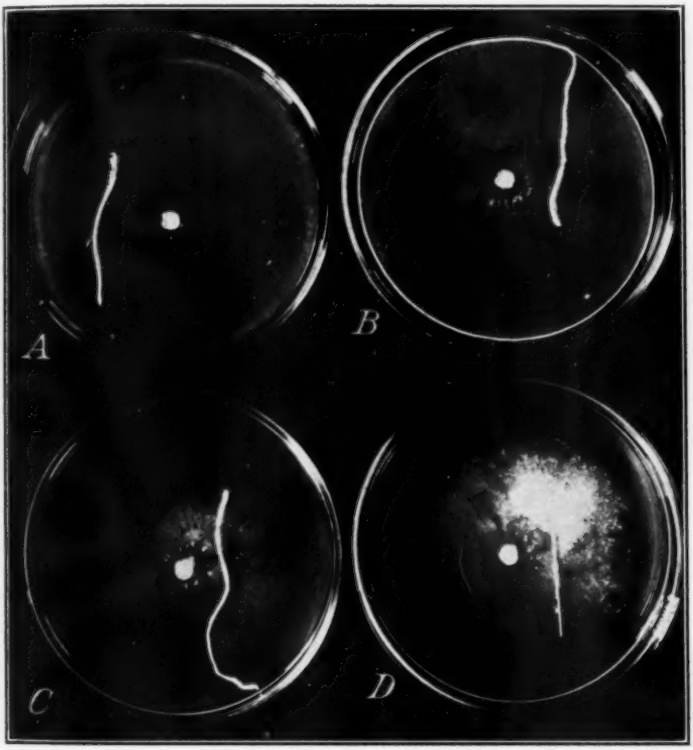

FIGURE 3.—Effect of different nutrients and auxins: *A*, Plain agar, inoculum, and root, no growth, *B*, agar, glucose, inoculum, and root, poor growth; *C*, agar, dihydrogen potassium phosphate, glucose; inoculum, and root, poor growth; *D*, agar, the essential salts, glucose, inoculum, and root, good growth.

to grow, the colony always remained fan-shaped. This is due to the fact that the mycelium absorbs and retains the growth-promoting substances as rapidly as they are diffused so that one-half of the plate which contains the root will also contain these substances and will, therefore, be able to support growth, while the opposite half, containing no root, will have no such substances and will, therefore, be unable to support growth. Even a casual examination of the colony will show that the richest growth of mycelium is made nearer the root, while the poorest is made in the immediate vicinity of the inoculum; with the exception of those substances which diffuse

through agar and reach the inoculum before any growth is made, all the available supply is absorbed and held by the growing mycelium nearer the source of supply. Even when the root is placed at one edge of the plate and the inoculum at the opposite edge, the picture remains the same, growth becoming more and more sparse as it is followed away from the root. When a strip of agar about 4 mm in width is cut away from the middle of the agar in the plate, leaving the two halves separated by a channel, and when these two halves are connected by an inoculum disk cut from a Petri-dish colony of the fungus by means of a cork borer, growth will be in that half of the agar which contains the root; the other half will show no growth because the young hyphae growing out of the inoculum disk will not allow the growth-promoting substances to diffuse through the inoculum disk into the opposite half of the agar.

It is not at all necessary to have a living root. Roots autoclaved at 15 pounds' pressure for one-half hour and then transferred to the nutrient solution induce a very good growth. When a piece of root is crushed in 15 cc of nutrient solution, excellent growth follows; yet, according to Thimann (*13*), plant tissues when crushed in water do not yield appreciable amounts of auxins. If the roots are cut and allowed to dry into a brittle, brown tissue and are then transferred to a flask of nutrient solution, again a good growth results. Popov (*9*) also found that the active principle remained undiminished in dried plants. Thimann, on the other hand, states that root tips of *Avena* do not continue to produce growth substances when cut off and that such substances are easily oxidized and destroyed. The writer wonders if the coleoptile of *Avena* is a very good subject upon which to test auxins, or if a mere curvature and a slight elongation of the shoot is a convincing index for measuring the concentration of auxins. In order to test the effect of oxidation still further, roots were placed in a 1 percent solution of potassium permanganate for 12 hours, then taken out, thoroughly washed, and transferred to flasks containing the nutrient solution. In all cases a good growth followed.

Growth-promoting substances from the corn root can withstand very rough treatments. Some roots were kept for 24 hours in 95-percent alcohol, in full-strength acetone, in chloroform, and in 1-percent solutions of lead nitrate, aluminum nitrate, picric acid, gold chloride, zinc nitrate, and the salts of some other heavy metals known to be strong protein precipitants. After they were washed thoroughly in three changes of sterile distilled water and transferred to the nutrient solution, they induced good growth in the fungus. However, 0.1 percent of bichloride of mercury, 1-percent solutions of copper sulphate and silver nitrate, and full-strength formalin (40 percent) seemed to destroy or inactivate the active principle. It was suspected that the toxic substances were not washed out entirely and that they diffused from the root and inactivated the fungus. Consequently, when the roots treated with bichloride of mercury, copper sulphate, and silver nitrate were first subjected to hydrogen sulphide for 6 hours, boiled in water until all odor of hydrogen sulphide disappeared, and then transferred to the nutrient solution, they induced growth once more. Similarly, when the roots treated with formaldehyde were boiled in distilled water and then transferred to nutrient solution, they gave off enough substances to induce a fairly good growth.

The auxins of the corn root were not destroyed when the roots were boiled in 20-percent sulphuric acid until they began to disintegrate, and were then washed in distilled water and transferred to the nutrient solution. A 12-hour exposure of the roots to N/10 sodium hydroxide solution failed to destroy these substances. A 12-minute exposure to the largest X-ray tube was without any effect (140 pre-voltage, 5 amperes, distance 10 inches).

Adsorption on charcoal, fuller's earth, silica gel, etc., has given good results in the separation of the different fractions of vitamin B and of the so-called "bioses." The writer employed 4-percent norit, an excellent adsorbing agent. A number of flasks, each containing 15 cc of the nutrient solution, were prepared. Each flask contained a piece of root about an inch in length. Ten days later the roots were removed, the solutions combined, and then divided into two equal lots. The first was kept as a check; the second was boiled with 4-percent norit until nearly dry and then made up to the original

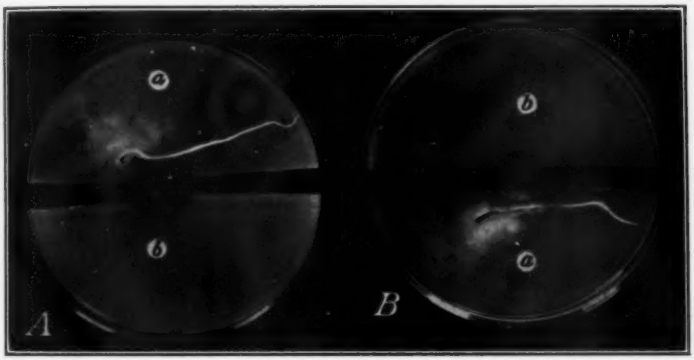

FIGURE 4.—*A* and *B*, Effect of adsorption. The black region in the agar consists of nutrient agar to which 4 percent of norit was added. The auxins from the root diffuse in all directions but because they are adsorbed on the norit barrier, inoculum *b* cannot grow, while inoculum *a*, having access to a supply of auxins, grows readily.

volume and filtered. Both the filtrate and the check were autoclaved, poured in a series of sterile glass capsules (preparation dishes), and inoculated. The checks induced a good growth, while the solution treated with norit induced no growth at all, showing that the growth factors were adsorbed on the norit.

The effect of norit was tested also in solid media. The nutrient solution was solidified by means of 2-percent agar-agar. The medium was poured in Petri dishes; after it had solidified, a strip of agar was cut away from the middle of the plate, thus separating the two halves of the nutrient agar by means of a channel. Another portion of the medium, to which 4 percent of norit was added, was poured in this channel. One primary root of corn was placed near this strip of norit-containing agar. The two halves of the untreated agar were then inoculated as indicated in figure 4. It can be seen that the growth factors which diffused through the base of the root were adsorbed on the norit and therefore failed to cross the channel and reach the inoculum *b*; consequently this inoculum failed to grow, while inoculum *a* made a good growth.

The effect of norit-treated auxin solutions upon sexuality of *Phytophthora cactorum* was tested. The writer has already demonstrated that a substance may be able to induce growth, but it may not, necessarily, be able to induce sexuality in this fungus. Proteose peptone is such a substance. When 0.2 percent of proteose peptone is added to the nutrient solution described in a former paragraph, it induces a very good growth but no sexual reproduction. A number of colonies grown in this solution were thoroughly washed in sterile distilled water and transferred to three different solutions, as follows: (1) Nutrient solution containing no auxins; (2) nutrient solution containing auxins; and (3) nutrient solution containing auxins, but heated with 4 percent of norit and filtered. The first solution did not induce any sexual bodies, while the second and the third did. It should be recalled that the second solution induced growth, while the third failed to do so. This indicates that while growth-promoting substances are adsorbed on norit, the sexuality-promoting factors remain unaffected.

NATURE OF THE GROWTH-PROMOTING SUBSTANCES

The term "auxin" is used reservedly in this paper because, while the writer believes that the growth-promoting factors diffused from the corn root are auxins, he has not obtained them in chemically pure form, and the evidence, therefore, is circumstantial in nature. Although ashed peas failed to induce growth, the objection might still be raised that ashing and the subsequent treatment with hydrochloric acid change the various minerals to chlorides and that such minerals may be active only in combination with some organic substances. In cooperation with an organic chemist, Dr. Virgil Lilly, the writer has conducted extensive investigations with growth-promoting substances of the garden pea. Ether and alcoholic extractions containing no minerals whatever have retained all the growth-promoting ability of the original substance. It is, therefore, quite safe to rule out inorganic salts. Similarly, carbohydrates, fats, proteins, proteoses, and amino acids have been left behind without in any way affecting the ability of the refined product to induce as good growth as the original substance.

The substance from the corn root cannot be considered of enzymic nature, for enzymes cannot withstand the violent treatments to which the corn roots were subjected without losing their growth-promoting ability. Nor is it likely that these substances are vitamins. Of the water-soluble types, vitamins B and G are the only ones likely to induce growth. Vitamin B_1 does not resist repeated autoclaving or the action of strong chemicals. The extract from the garden peas which Dr. Lilly and the writer have prepared, purified, and tested, were used by Hazel C. Cameron of this station in some vitamin-G-free diet feeding experiments with rats. The results were all negative, and it is safe to conclude that the growth-promoting factor is not vitamin G.

The growth-promoting substances from the corn roots are not pantothenic acid. The crystalline pantothenic acid obtained through the courtesy of Dr. R. J. Williams failed to induce any growth in *Phytophthora cactorum*. Similarly, one of his "nutrilites" failed in

this respect. Pantothenic acid is a good growth-promoting substance for yeasts; "nutrilite 103" promotes growth in some other fungi with which Dr. Williams has experimented. It would, therefore, be an idle generalization to assume that any one auxin may promote growth in all fungi. As previously stated, Kögl and his coworkers have isolated three different auxins from human urine. The one extracted from the garden pea by Dr. Lilly and the writer is different in its chemical properties from auxin A, B, and heteroauxin. There is no reason why there should not be a great many different auxins in nature.

Mitogenetic radiation can at once be waved aside because such radiations, if they actually exist, are associated with living tissues. Corn roots killed by mechanical means, by chemical treatments, and by heat still retain their growth-promoting ability. Furthermore it is difficult to conceive that such rays would be stopped by a narrow strip of agar containing norit (fig. 4).

The definite polarity of the growth factor in the corn root is identical with the behavior of auxins of other investigators. It is not probable that ordinary minerals or organic substances manifest such behavior.

In the light of the foregoing discussion the writer is inclined to favor the theory that the growth-promoting substances present in the corn roots are auxins.

SUMMARY

Canned peas induce an excellent growth and reproduction in *Phytophthora cactorum*. When, however, the peas are ashed, and the ash is added to a mineral solution plus a pure grade of dextrose, the fungus does not grow. But if a piece of root about an inch in length is cut from an aseptically sprouting corn grain and transferred to this solution, the fungus makes a very good growth and reproduces normally.

The root does not need to remain in the solution for very long. Even as short an exposure as 1 minute suffices to endow the solution with growth-promoting properties; however, the growth is richer with the longer exposures.

The growth-promoting substances in the root move toward the base; consequently only very few auxins are given off from the apical portion even when the root tip is cut off. The amount given off from the sound roots is negligible.

The growth-promoting substances as given off by the cut root are unable to induce growth without the essential salts and sugar.

It is not necessary to have living roots. Crushed or autoclaved roots induce excellent growth. Many toxic substances and protein precipitants are without effect; X-rays do not exert any harmful influence.

Growth-promoting factors are adsorbed on norit, so that a norit-treated solution no longer promotes growth. Sexuality-promoting substances, on the other hand, are not adsorbed by norit, so that norit-treated auxin solutions promote sexuality just as well as the untreated ones. This shows that in addition to growth-promoting substances there are sexuality-promoting factors which, apparently possess different chemical and physical properties.

LITERATURE CITED

(1) ALMOSCHLECHNER, E.
1934. DIE HEFE ALS INDIKATOR FÜR WUCHSTOFFE. Planta Arch. Wiss. Bot. 22: [515]–542, illus.

(2) BUCHANAN, R. E., and FULMER, E. I.
1928–30. PHYSIOLOGY AND BIO-CHEMISTRY OF BACTERIA. 3 v., illus. Baltimore.

(3) CHOLODNY, N.
1934. UEBER DIE BILDUNG UND LEITUNG DES WUCHSHORMONS BEI DEN WURZELN. Planta Arch. Wiss. Bot. 21: 517–530, illus. [Abstract in Chem. Abs. 28: 6771. 1934.]

(4) EULER, H. v., and PHILIPSON, T.
1932. WASSERLÖSLICHE WACHSTUMFAKTOREN. Biochem. Ztschr. 245: [418]–430. [Abstract in Chem. Abs. 26: 3281. 1932.]

(5) KÖGL, F., HAAGEN-SMIT, A. J., and ERXLEBEN, H.
1932. ÜBER EIN PHYTOHORMON DER ZELLSTRECKUNG. REINDARSTELLUNG DES AUXINS AUS MENSCHLICHEM HARN. 4. MITTEILUNG ÜBER PFLANZLICHE WACHSTUMSSTOFFE. Hoppe-Seyler's Ztschr. Physiol. Chem. 214: 241-261, illus.

(6) MILLER, W. L.
1930. BIOS. Jour. Chem. Ed. 7: 257–267.

(7) NIELSEN, N.
1931. THE EFFECT OF RHIZOPIN ON THE PRODUCTION OF MATTER OF ASPERGILLUS NIGER. Compt. Rend. Lab. Carlsberg v. 19, no. 5, 10 pp.

(8) ——— and HARTELIUS, V.
1932. THE SEPARATION OF GROWTH-PROMOTING SUBSTANCES. Compt. Rend. Lab. Carlsberg 19, no 8, 17 pp.

(9) POPOFF, M. [POPOV, M.]
1933. UEBER DIE PFLANZLICHEN AUXINE UND IHRE WIRKUNG AUF EINZELLIGE. Biol. Zentbl. 53: 661–669. [Abstract in Chem. Abs. 28: 1073. 1934.]

(10) SCHOPFER, W. H.
1934. VERSUCHE ÜBER DIE WIRKUNG VON SEINEN KRISTALLISIERTEN VITAMINEN B AUF PHYCOMYCES. Ber. Beut. Bot. Gessell. 52: 308-312.

(11) SHERMAN H. C., and SMITH, S. L.
1931. THE VITAMINS. Ed. 2, 575 pp., illus. New York.

(12) TANNER, F. W.
1925. THE "BIOS" QUESTION. Chem. Rev. 1: 397-472.

(13) THIMANN, K. V.
1934. STUDIES ON THE GROWTH HORMONE OF PLANTS. VI. THE DISTRIBUTION OF THE GROWTH SUBSTANCE IN PLANT TISSUE. Jour. Gen. Physiol. 18: 23–34, illus.

(14) WENT, F. W.
1928. WUCHSSTOFF UND WACHSTUM. Rec. Trav. Bot. Néerlandais 25: 1–114, illus.

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